BRANCHED DNA NANOSTRUCTURES FOR MOLECULAR DIAGNOSTICS

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Mark Richard Hartman

August 2013

BRANCHED DNA NANOSTRUCTURES FOR MOLECULAR DIAGNOSTICS

Mark Richard Hartman, Ph.D.

Cornell University 2013

DNA nanotechnology has been enormously successful in using DNA as a nanoscale construction material, and consistent progress in using increasingly complex DNA-mediated assemblies and designs has been reported in the literature. However, the field of DNA nanostructures has sometimes been lacking for concrete applications. Meanwhile, DNA has also been widely used as probes and primers for nucleic acid diagnostics applications, and this provides an extremely critical application with potential for great medical benefits, and these DNA probes and primers are readily interfaced with DNA nanotechnology. Thus, in this Dissertation, I discuss the combination of (1) DNA in its role as a recognition element for nucleic acid detection, and (2) DNA as a structural material for nanoscale self-assembly. This combination represents a fusion of "structure" and "function", and will ultimately lead to great advancements in the field of nucleic acid diagnostics.

BIOGRAPHICAL SKETCH

I received my B.S. degree in Chemical and Biomolecular Engineering from Cornell University in 2007. As an undergraduate with a minor in Biological Engineering, I worked with Prof. Dan Luo in the Biological and Environmental Engineering on the design and synthesis of DNA nanostructures and portable detection using DNA nanobarcodes. Upon graduation, I remained at Cornell and entered the Masters of Engineering program (2007 – 2008), where I worked on DNA-mediated self-assembly of gold nanoparticles. I continued my graduate studies towards a Ph.D. in the Department of Biological and Environmental Engineering at Cornell University, still working in the laboratory of Prof. Dan Luo. During my tenure in the Luo lab, I investigated the use of DNA as both a generic and genetic material, and developed skills for engineering DNA nanostructures with a focus towards diagnostic applications.

To my family and friends

ACKNOWLEDGEMENTS

My Ph.D. degree would not have been possible without the support through the years from Professor Dan Luo, my academic advisor and chair of my committee. I am also grateful to my committee members Professor John March and Professor Edwin Kan for their advice and for making themselves available whenever I needed their help. I would like to thank Dr. Nokyoung Park and Dr. Wenlong Cheng for working with me during my early years in LuoLabs. I would also like to thank Dr. Thua Tran for constant willingness to provide helpful advice and guidance. Many thanks go out to all the other members of LuoLabs who were always willing to share their advice and laboratory supplies. Specifically, I would like to thank Edward Rice, Mike Campolongo, Shawn Tan, Jason Kahn, Natt Kiatwuthinon, Ken Yancey, Dayong Yang, Roanna Ruiz, Songming Peng, Hoon Roh, Tom Derrien, and Jong Bum Lee.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

In this dissertation, I will utilize DNA nanostructures in molecular diagnostics to demonstrate how the unique advantages of DNA nanostructures, including multifunctionality and precise controllability, can be leveraged for nucleic acid detection applications. In this introduction, I will discuss how the structural properties of DNA have enabled the development of progressively more complex DNA nanostructures. Next, I will explore how the biological importance of nucleic acids has led to the burgeoning field of molecular diagnostics for research and clinical applications. Finally I will outline how the integration of DNA nanostructures can lead to significant improvements for existing molecular diagnostic techniques.

1.1 DNA nanostructures

In recent decades, DNA has gained attention as an excellent structural material for nanometer-scale engineering. As illustrated in Table 1.1, DNA has several unique properties that motivate its use for engineering nanostructures.

Ease of handling	• Facile synthesis • Chemical stability • Simple modification
Biological significance	• DNA-processing enzymes • Important applications in delivery and diagnostics
Predictable behavior	• Watson-Crick base pairing • *in silico* modeling

Table 1.1: Summary of DNA's attributes, which make it attractive as a material for structural engineering at the nanometer scale.

Firstly, DNA is easy and convenient both to synthesize and to handle in the laboratory. Single-stranded DNA oligonucleotides can be synthesized commercially at low cost with reliable yield and purity. The chemical structure of DNA is consistent and well-controlled; in particular, in double-stranded DNA the position of any specific base placement is effectively controlled with a resolution of approximately 0.34 nanometers. Additionally, with appropriate handling DNA is extraordinarily stable, especially when lyophilized (e.g. as demonstrated by the genetic analysis of ancient mummified human remains). This stability reflects its natural role as the primary biological information storage medium. Conditions in the laboratory can be controlled to prevent DNA degradation or spontaneous mutation as a result of oxidative damage or

radiation. Furthermore, a variety of chemical and bio-functional modifications, such as fluorescent labels, quenchers, biotin for further functionalization, and chemical groups, can be appended to synthetic DNA oligonucleotides. These moieties may enable specific functions as well as further modification.

Secondly, DNA has substantial biological significance. The vital role of DNA in fundamental biological processes has resulted in the co-evolution of a wide variety of DNA-associated enzymes that can manipulate and process. The possibility of enzymatic processing further expands the versatility and controllability of DNA as an engineering material. The biological relevance of DNA has also led to important applications such as molecular diagnostics (the focus of this dissertation). It must be noted that this biological significance is not entirely unique to DNA, but is also true of other biopolymers including proteins and carbohydrates.

The third important property of DNA, which motivates its use in nanostructural engineering and distinguishes it from the other biopolymers, is its predictability. DNA follows Watson Crick base-pairing rules. DNA forms secondary structures that are complex but can frequently be modeled *in silico*.[1] In contrast, structural prediction of proteins or carbohydrates is more complicated, and thus engineering nanostructures with these materials has been prohibitively difficult.

The predictability of DNA has allowed for a large variety of diverse structures to be designed and engineered with confidence, and a number of software packages have been extraordinary successful in this regard.

The DNA branched junction

The basis for engineering DNA nanostructures is the DNA branched junction. DNA is typically known to form linear and circular structures; however, it can also form branches. This was first understood with studies of genetic recombination, where the four-armed X-shaped DNA branch became known as the Holliday junction after Robin Holliday, who proposed it in 1964.[2] Soon additional types of branched DNA structures became known. Seeman and coworkers pioneered the first stable, synthetic branched DNA nanostructures by designing partially-complementary oligonucleotides that could form various junctions such as X-shaped and Y-shaped branched DNA[3–5] and more complex shapes such as a cube[6] and an octahedron[7]. This work also led to development of double-crossover DNA (discussed in next section of this thesis).[8]

In 2003, Luo laboratory published work on dendrimer-like DNA, which used branched structures as basic building blocks, and combined these building

blocks into more complex structures via enzymatic ligation (taking advantage of DNA-processing enzymes).[9] This work was further developed through the production of an enzyme-catalyzed DNA hydrogel,[10] DNA nanobarcodes,[11], [12] and ABC monomers,[13] leading to applications in detection and cell-free protein production[14], [15]. Meanwhile other workers have utilized branched DNA to generate bulk scale DNA hydrogels and dendrimers based entirely on DNA hybridization without the use of enzymes [16], [17].

Double crossover DNA

As work continued on simple branched DNA nanostructures, other workers focused on slightly more complex basic structures for DNA building blocks: double crossover DNA. The basic design for double crossover DNA was first popularized by Seeman.[18] Later works showed its usage in complex tiling and patterned structures, which led to a large number of impressive demonstrations.[19] Importantly, the use of double crossover DNA allows for DNA nanostructures that are rigid and well-defined, in contrast to simple branched DNA nanostructures that are relatively flexible.

The DNA crossover design was used as the underlying scaffold for DNA origami, which utilized very long single-stranded DNA (the template strand,

such as M13 phage DNA) that was linked together into a pre-defined structure using a large number of shorter single-stranded DNA ("staple strands").[20] DNA origami has allowed an unprecedented level of control over custom DNA nanostructures and has become an extremely popular approach for design and construction of a large variety of synthetic DNA nanostructures. More recently, this strategy has also been used in a "molecular canvas" approach, which is similar to DNA origami but utilizes many short strands of DNA without any long single-stranded template strand.[21]

1.2 Molecular diagnostics

Nucleic acids are extremely important biomarkers and can be useful indicators of disease. Pathogens carry genetic information that can be specifically recognized and identified as foreign relative to the healthy disease-free organism. Expressed messenger RNA (mRNA) can be profiled to give information about the current state of the organism, tissue, or cell, and thus provides a "bird's eye view" of the functioning of a biological system. The genetic code can be considered as the original source for information transmission in the organism, and thus can be useful for clinical prognostics and for the diagnosis of non-infectious diseases such as autoimmune diseases or cancer.

The biological importance of nucleic acids, as the "information medium of life", has led to the growth and development of the molecular diagnostics field. Historically, protein/antibody-based diagnostics were the most popular basis for *in vitro* disease detection. However, as of this writing in 2013, nucleic acid-based diagnostics are growing in importance largely due to the high expectations raised by next generation sequencing. Nucleic acid-based detection has crucial advantages relative to immunologically-based tests: its compatibility with exponential enzymatic amplification, its simplicity and predictability (DNA primers and probes can be synthesized quickly and easily whereas new antibodies must be raised through a longer process), and its ability to distinguish specific genetic markers that may be difficult or impossible to detect through immunological means. As a result of exponential enzymatic amplification, sensitivity of nucleic-acid based tests is generally higher than immunologically based tests – PCR can technically amplify even a single molecule of target DNA or RNA in a pathogenic sample. As a result of this higher sensitivity, PCR-based assays are a popular approach for certain clinical scenarios (e.g. viral load testing for HIV).

In this introduction, "molecular diagnostics" is defined to encompass at least three broad methods, each of great significant for clinical medicine: nucleic acid tests (enzymatic amplification including PCR and isothermal amplification

techniques), gene expression profiling (DNA microarrays), and DNA sequencing.

Nucleic acid tests for detection

Currently almost all molecular diagnostics make use of PCR. PCR takes advantage of the natural genetic replication of DNA for *in vitro* amplification. Traditionally, PCR utilizes a thermostable DNA polymerase enzyme (such as Taq) and thermal cycling to carry out multiple rounds of successive denaturation, annealing, and extension. Successful amplification requires primers that are specifically designed to bind to a template strand (which may also be referred to as the target strand). During the PCR process, every cycle yields a two-fold increase in the amount of product nucleic acid, resulting in exponential amplification. Theoretically, this amplification takes place only in the presence of the template strand. Thus, the "output" of the PCR is a large amount of a specific DNA product, which is traditionally characterized using gel electrophoresis.

Upon its development, PCR rapidly became of great importance in almost all areas of molecular biology, and was very popular and useful for detection applications. However, traditional PCR is unsuitable for accurate quantitative

measurements of target DNA in an unknown sample, because large amounts of DNA product are generated whether the original amount of target DNA is large or small. This issue was resolved by the development of quantitative PCR (qPCR). In a typical qPCR process, the amount of product is monitored as it occurs in real time, through the use of a DNA-specific dye, molecular beacon, or similar technique. Considering the number of cycles required to achieve a certain amount of PCR product, and the amplification rate of the PCR process, qPCR results in a more accurate estimate of the original amount of DNA template. Thus qPCR can be extremely valuable for making diagnoses when quantitative, rather than qualitative, results are beneficial or necessary.

Aside from the lack of quantitative information, another disadvantage of traditional PCR is the time and equipment (and associated electrical energy requirement) needed for thermal cycling. The requirement for thermal cycling can be avoided through the use of isothermal amplification techniques. Many different enzyme-based isothermal amplification techniques are possible, including rolling circle amplification (RCA), loop mediated amplification (LAMP), helicase-dependent amplification (HDA), and nucleic acid based sequence amplification (NASBA), as well as many others. These technologies are functionally similar to PCR, using enzymes to amplify the template or target DNA strand. Typically, these isothermal amplification techniques are more

complex than PCR, requiring additional enzymes or primers, and relatively complicated primer design. (In effect, the "procedural complexity" represented by thermal cycling is exchanged for "chemical complexity" in terms of the components of the reaction.) Currently, PCR remains the "gold standard" for nucleic acid testing, too widely-used and well-understood to be replaced by any isothermal technique, except in niche applications such as point-of-care diagnostics.

Gene expression profiling

The broad goal of gene expression profiling is to identify and quantify the activity of many genes (on the order of thousands) that are being expressed by a given organism, tissue, or cell. A typical expression profiling experiment may utilize enzymatic amplification as part of the process. The traditional approach to carry out gene expression experiments is using hybridization-based DNA microarrays. In this approach, single-stranded "capture probe" oligonucleotides are attached to a solid support. Each capture probe hybridizes to a specific nucleic acid that may be present in the test sample, and this hybridization event triggers a specific measureable response. By assessing the amount of each type of

mRNA present in the sample, a global picture of cellular function can be obtained.

DNA microarrays are the traditional tool for gene expression profiling, but many alternative formats are possible. The Nanostring© platform has gained attention as a successful method to profile individual mRNA molecules using fluorescence microscopy.[22] In addition, as next generation sequencing has grown in popularity, sequencing-based methods for gene expression profiling have emerged as an alternative to more DNA microarray-based approaches. These sequencing-based expression profiling methods may be referred to as RNA-Seq or transcriptome sequencing.

DNA sequencing

Next generation sequencing (NGS) has proved extremely useful and is currently growing in importance for both research and clinical applications.[23] For a typical NGS experiment, the target nucleic acid is fragmented and amplified to form identical copies of different fragments. Each fragment is sequenced in parallel with data being collected from all sequencing reactions simultaneously. The sequencing data collected from each fragment is aligned and assembled

together, either using an existing known sequence as a scaffold (re-sequencing) or without any pre-existing information (de novo sequencing).

The increasing prominence of next generation sequencing has important implications for personalized medicine. As the cost and time required for sequencing is reduced, it is becoming increasingly practical to sequence the genome for every individual patient to obtain predictive information about their genetic predisposition to disease and compatibility with specific treatments. In the case of cancer, it is desirable to sequence tumor cells to assess the susceptibility of a specific tumor for alternative treatments. Thus, NGS is part of the new standard of care for cancer, and will certainly play a prominent role in the future of healthcare.

1.3 DNA nanostructures for molecular diagnostics

Traditional molecular diagnostics do not take advantage of DNA nanostructures. However, traditional molecular diagnostic methods do require many DNA-based components (primers and probes). These can be potentially replaced or modified with DNA nanostructures such as branched DNA. This is an excellent opportunity to introduce novel characteristics, which are attainable by DNA nanostructures but not by conventional linear primers and probes, such as

anisotropic multifunctionality and precise controllability, which may introduce important capabilities and advantages for detection.

As one example of branched DNA probes, Um and coworkers developed DNA-based "fluorescent nanobarcodes" to simultaneously identify multiple pathogen DNA sequences [11], [12]. Each nanobarcode encoded a unique identifier, something like a common supermarket barcode. In the presence of pathogen DNA sequences, these nanobarcodes hybridized to the target DNA and were detected by fluorescence microscopy. Based on the fluorescent color ratio of each aggregate, the identity of pathogen DNA was easily determined. This strategy uses "reaction encoding", with the pathogen identity contained within the label itself, rather than "spatial encoding" as used by conventional DNA microarrays. Using multiple types of nanobarcodes, each complementary to a different pathogen DNA sequence, many different pathogens could be detected simultaneously.

Another example of branched DNA for sensing is the so-called branched DNA (bDNA) assay which uses branched DNA to amplify the signal from a target DNA strand. This strategy is similar to the enzyme-linked immunosorbent assay (ELISA), in which the signal from a primary antibody is amplified by a multi-labeled secondary antibody; however, hybridization between complementary

DNA sequences replaces the specific antibody-antigen interactions in ELISA. After hybridization based-amplification, the resulting branched structures are labeled with an enzyme to generate an amplified signal.[24] This approach has been successfully commercialized for a number of clinical applications, but its popularity is limited relative to established approaches such as PCR.

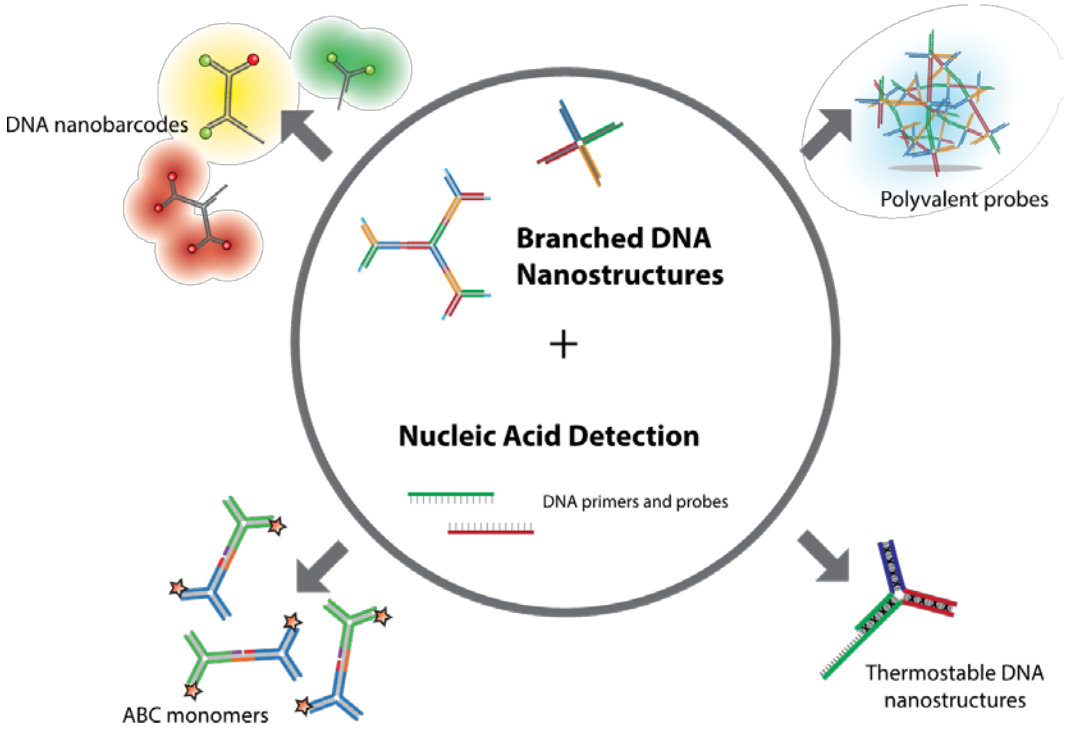


Figure 1.1: Scheme illustrating the combined roles of nucleic acids for detection with DNA nanotechnology-enabled structures for the development of novel diagnostics.

1.4 Significance of this dissertation

DNA nanostructures can be directly implemented into existing approaches for molecular diagnostics. The key consideration is whether the added benefits of DNA nanostructures (multifunctionality and controllability) outweigh the added complexities of synthesizing and integrating DNA nanostructures into existing strategies. I argue that integrating DNA nanostructures into existing molecular diagnostics will have many potential benefits, the added complexity of DNA nanostructures is low, and the possibility of implementing DNA nanostructures into existing molecular diagnostics is a rich area for exploration and should be seriously considered. With the rise of NGS and growing popularity of molecular diagnostics, it is increasingly important to thoroughly explore these options.

In this dissertation I explore two important concepts that are related to the use of DNA nanostructures in diagnostic applications. Chapter 2 discusses the stability of DNA nanostructures. Despite the great advantages of utilizing DNA nanostructures as probes and/or primers, DNA nanostructures have the fundamental limitation that they are denatured under harsh conditions. As a result, DNA nanostructures typically cannot be used in any applications that involve denaturing conditions (high temperatures, low salinity, high pH, and

presence of certain chaotropic chemicals), and this limits their real-world applications including their use in diagnostics. I have aimed to address this limitation in a simple and direct manner by utilizing a chemical cross-linking method that creates covalent bonds between double-stranded DNA and results in a stabilized (thermostable) structure. This enables DNA nanostructures to be used as probes or primers even in high temperature applications such as PCR. Chapter 3 discusses the concept of point-of-care nucleic acid detection, including an overview of the field and prominent examples from the literature. Chapter 4 addresses the issue of hybridization kinetics. Surface-based hybridization is fundamentally slower than solution phase hybridization, and also requires specific functionalization and treatment of surface chemistry to enable desired interactions and prevent fouling. I propose the use of solution-based hybridization for detection that is more rapid and avoids the requirement of surface modification. The significance of this work is discussed in regards to point-of-care diagnostics, with specific considerations on applying this work toward resource-limited settings such as the developing world.

1.5 References

[1] J. SantaLucia and D. Hicks, "The thermodynamics of DNA structural motifs.," *Annu Rev Biophys Biomol Struct*, vol. 33, pp. 415–40, Jan. 2004.

[2] Y. Liu and S. C. West, "Happy Hollidays: 40th anniversary of the Holliday junction.," *Nat Rev Mol Cell Biol*, vol. 5, no. 11, pp. 937–44, Nov. 2004.

[3] R. I. Ma, N. R. Kallenbach, R. D. Sheardy, M. L. Petrillo, and N. C. Seeman, "Three-arm nucleic acid junctions are flexible.," *Nucleic Acids Res*, vol. 14, no. 24, pp. 9745–53, Dec. 1986.

[4] N. R. Kallenbach, R.-I. Ma, and N. C. Seeman, "An immobile nucleic acid junction constructed from oligonucleotides," *Nature*, vol. 305, no. 5937, pp. 829–831, Oct. 1983.

[5] N. C. Seeman and N. R. Kallenbach, "Design of immobile nucleic acid junctions.," *Biophys J*, vol. 44, no. 2, pp. 201–9, Nov. 1983.

[6] J. H. Chen and N. C. Seeman, "Synthesis from DNA of a molecule with the connectivity of a cube.," *Nature*, vol. 350, no. 6319, pp. 631–3, Apr. 1991.

[7] Y. Zhang and N. C. Seeman, "Construction of a DNA-Truncated Octahedron," *J Am Chem Soc*, vol. 116, no. 5, pp. 1661–1669, Mar. 1994.

[8] N. C. Seeman, "DNA in a material world.," *Nature*, vol. 421, no. 6921, pp. 427–31, Jan. 2003.

[9] Y. Li, Y. D. Tseng, S. Y. Kwon, L. D'Espaux, J. S. Bunch, P. L. McEuen, and D. Luo, "Controlled assembly of dendrimer-like DNA.," *Nature Materials*, vol. 3, no. 1, pp. 38–42, Jan. 2004.

[10] S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach, and D. Luo, "Enzyme-catalysed assembly of DNA hydrogel.," *Nature Materials*, vol. 5, no. 10, pp. 797–801, Oct. 2006.

[11] Y. Li, Y. T. H. Cu, and D. Luo, "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes.," *Nature Biotechnology*, vol. 23, no. 7, pp. 885–9, Jul. 2005.

[12] S. H. Um, J. B. Lee, S. Y. Kwon, Y. Li, and D. Luo, "Dendrimer-like DNA-based fluorescence nanobarcodes.," *Nat Protoc*, vol. 1, no. 2, pp. 995–1000, Jan. 2006.

[13] J. B. Lee, Y. H. Roh, S. H. Um, H. Funabashi, W. Cheng, J. J. Cha, P. Kiatwuthinon, D. A. Muller, and D. Luo, "Multifunctional nanoarchitectures from DNA-based ABC monomers.," *Nature Nanotechnology*, vol. 4, no. 7, pp. 430–6, Jul. 2009.

[14] N. Park, S. H. Um, H. Funabashi, J. Xu, and D. Luo, "A cell-free protein-producing gel.," *Nature Materials*, vol. 8, no. 5, pp. 432–7, May 2009.

[15] N. Park, J. S. Kahn, E. J. Rice, M. R. Hartman, H. Funabashi, J. Xu, S. H. Um, and D. Luo, "High-yield cell-free protein production from P-gel.," *Nature Protocols*, vol. 4, no. 12, pp. 1759–70, Jan. 2009.

[16] Y. Xing, E. Cheng, Y. Yang, P. Chen, T. Zhang, Y. Sun, Z. Yang, and D. Liu, "Self-assembled DNA hydrogels with designable thermal and enzymatic responsiveness.," *Advanced Materials*, vol. 23, no. 9, pp. 1117–21, Mar. 2011.

[17] T. Zhou, P. Chen, L. Niu, J. Jin, D. Liang, Z. Li, Z. Yang, and D. Liu, "pH-responsive size-tunable self-assembled DNA dendrimers.," *Angew Chem Int Ed Engl*, vol. 51, no. 45, pp. 11271–4, Nov. 2012.

[18] X. Li, X. Yang, J. Qi, and N. C. Seeman, "Antiparallel DNA Double Crossover Molecules As Components for Nanoconstruction," *J Am Chem Soc*, vol. 118, no. 26, pp. 6131–6140, Jan. 1996.

[19] E. Winfree, F. Liu, L. A. Wenzler, and N. C. Seeman, "Design and self-assembly of two-dimensional DNA crystals.," *Nature*, vol. 394, no. 6693, pp. 539–44, Aug. 1998.

[20] P. W. K. Rothemund, "Folding DNA to create nanoscale shapes and patterns.," *Nature*, vol. 440, no. 7082, pp. 297–302, Mar. 2006.

[21] B. Wei, M. Dai, and P. Yin, "Complex shapes self-assembled from single-stranded DNA tiles.," *Nature*, vol. 485, no. 7400, pp. 623–6, May 2012.

[22] G. K. Geiss, R. E. Bumgarner, B. Birditt, T. Dahl, N. Dowidar, D. L. Dunaway, H. P. Fell, S. Ferree, R. D. George, T. Grogan, J. J. James, M. Maysuria, J. D. Mitton, P. Oliveri, J. L. Osborn, T. Peng, A. L. Ratcliffe, P. J. Webster, E. H. Davidson, L. Hood, and K. Dimitrov, "Direct multiplexed measurement of gene expression with color-coded probe pairs.," *Nat Biotechnol*, vol. 26, no. 3, pp. 317–25, Mar. 2008.

[23] A. S. Gargis, L. Kalman, M. W. Berry, D. P. Bick, D. P. Dimmock, T. Hambuch, F. Lu, E. Lyon, K. V Voelkerding, B. A. Zehnbauer, R. Agarwala, S. F. Bennett, B. Chen, E. L. H. Chin, J. G. Compton, S. Das, D. H. Farkas, M. J. Ferber, B. H. Funke, M. R. Furtado, L. M. Ganova-Raeva, U. Geigenmüller, S. J. Gunselman, M. R. Hegde, P. L. F. Johnson, A. Kasarskis, S. Kulkarni, T. Lenk, C. S. J. Liu, M. Manion, T. A. Manolio, E. R. Mardis, J. D. Merker, M. S. Rajeevan, M. G. Reese, H. L. Rehm, B. B. Simen, J. M. Yeakley, J. M. Zook, and I. M. Lubin, "Assuring the quality of next-generation sequencing in clinical laboratory practice.," *Nat Biotechnol*, vol. 30, no. 11, pp. 1033–6, Nov. 2012.

[24] A. N. Player, L.-P. Shen, D. Kenny, V. P. Antao, and J. A. Kolberg, "Single-copy Gene Detection Using Branched DNA (bDNA) In Situ Hybridization," *Journal of Histochemistry & Cytochemistry*, vol. 49, no. 5, pp. 603–611, May 2001.

CHAPTER 2

BRANCHED PCR USING THERMOSTABLE DNA NANOSTRUCTURES AS MODULAR PRIMERS

2.1 Introduction

The extraordinary controllability of synthetic DNA nanostructures offers great potential for nanotechnology and biotechnology,[1–5] with applications including self-assembly,[6–11] sensing,[12–15] and DNA-based materials.[16–19] However, DNA nanostructures are unstable at high temperatures due to the inherent thermal instability of base pairing, which limits their real-world applications. Recent work has demonstrated the use of psoralen, a naturally-occurring reagent that intercalates and cross-links DNA,[20] to create DNA origami structures with enhanced thermal stability.[21], [22] In addition, other thermostable bonds have been introduced in the form of DNA-organic molecule hybrids to construct DNA structures and networks.[23–25]

In this Chapter, I present a general approach using our previously-reported branched DNA building blocks[26], [27] combined with psoralen cross-linking to produce thermostable branched DNA nanostructures. These thermostable

branched DNA nanostructures can withstand denaturing conditions without disruption of their integrity. Importantly, this system is extremely modular. Simple nanostructures can be combined in a controlled fashion (via enzymatic ligation) to form increasingly complex nanostructures, and individual component oligonuleotides can be "swapped out" for different labelling and functions.

This unique modularity enabled us to utilize thermostable branched DNA nanostructures as modular primers in polymerase chain reaction (PCR), achieving novel "branched PCR." Our branched PCR maintains the controllability of branched DNA nanostructures, while preserving the biological function of the resulting PCR products. Furthermore, we demonstrate the novelty and utility of branched PCR through the realization of multifunctional labelling for detection, synthesis of branched PCR products, and hydrogel formation, none of which can be achieved by conventional PCR.

2.2 Approach

Our branched thermostable DNA structures were formed by a two-step process, as shown in Figure 2.1. In the first step, we synthesized Y-shaped DNA following our previously-reported protocol.[26] Briefly, three single-stranded DNA were

rationally designed with specific sequences such that each was partially complementary to another, resulting in self-annealed, branched, Y-shaped DNA. In the second step, the structures were incubated with psoralen and briefly exposed to UV illumination. DNA sequences were deliberately designed to yield a high proportion of interstrand cross-linking sites during this psoralen treatment step (Figure 2.2). Sequences are shown in Table 2.1.

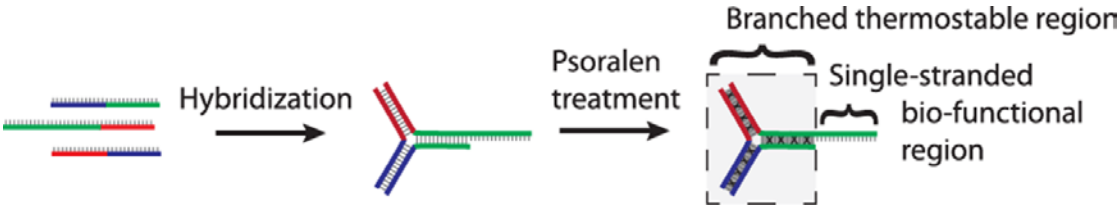


Figure 2.1: Illustration of cross-linking of branched DNA structures. Single-stranded oligonucleotides hybridize to form branched DNA nanostructures through DNA base-pairing. Resulting nanostructures are treated with psoralen to cause covalent interstrand cross-linking. Resulting structures are thermostable and will remain intact under denaturing conditions.

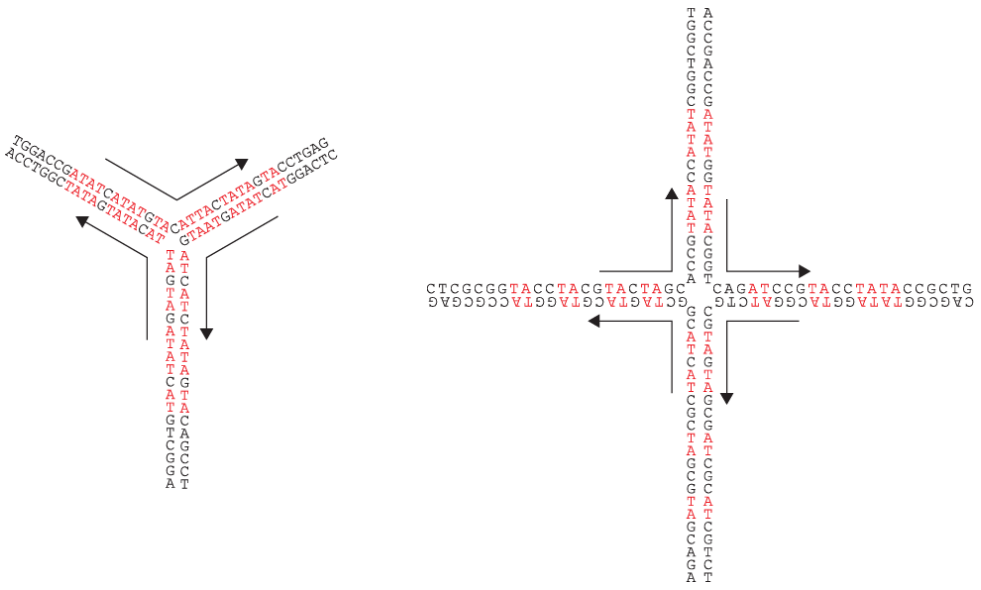


Figure 2.2: Diagram of sequences for Y-shaped and X-shaped DNA branches used in this work. Arrows indicate 5′ to 3′ polarity. Psoralen cross-linking is most likely to occur at 5′-TA-3′ and 5′-AT-3′ sites, which are indicated with red text. Varying the number of 5′-TA-3′ and 5′-AT-3′ sites changes the effective yield of cross-linked "thermostable" product.

After this simple treatment, the branched DNA nanostructures were thermostable and remained intact even under denaturing conditions. These thermostable branched DNA nanostructures were ideal for high temperature-based applications, such as PCR. To implement our structures in PCR, we simply substituted our modular primers in place of the standard linear primers, as shown in Figure 2.3. During the denaturing phase of PCR, the linear template

was denatured while the modular primers remained intact due to the cross-linking with psoralen. During the annealing and extension phases, the modular primers hybridized to the template and were extended by the polymerase, resulting in a "dumbbell-shaped" product. Exponential amplification occurred as usual, except that products were branched.

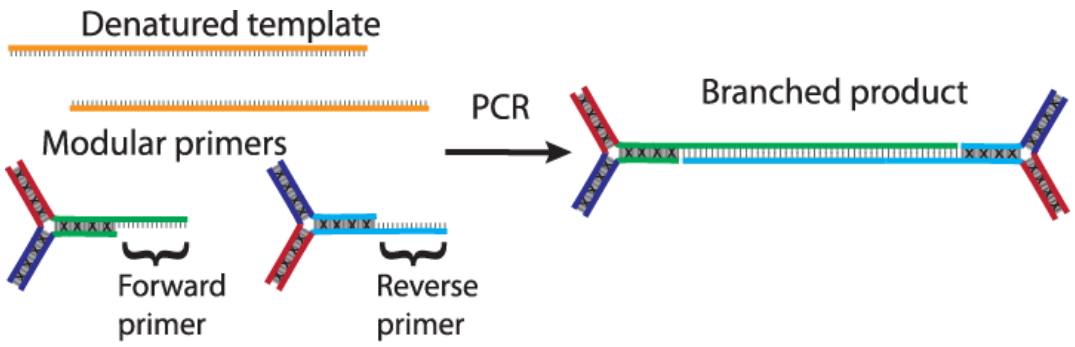


Figure 2.3: Illustration of cross-linked branched DNA structures for use in PCR. Cross-linked products can be used in PCR as modular primers, replacing the conventional linear primers, resulting in branched PCR products.

2.3 Experimental methods

Preparation of DNA nanostructures. Oligonucleotides were ordered from Integrated DNA Technologies (IDT) with sequences as shown below. Branched

Y-shaped and X-shaped DNA structures were prepared according to previously published methods from our group.[26] Briefly, branched DNA structures were synthesized by mixing the same molar amount of corresponding oligonucleotide strands. To form fluorescent tagged Y-shaped DNA, commercially synthesized fluorescent tagged oligonucleotide strands were used.

Oligonucleotide sequences. For experiments with modular primers, the primer sequences shown in Table 2.1 were appended directly to the 3' end of the desired core sequences. For experiments involving ligation, the single-stranded overhang sequences shown in Table 2.1 were added directly to the 5' end of the desired core sequences. For experiments with fluorescent Y-shaped DNA, FAM (green) or Cy5 (red) modifications were added directly to the 5' end of the desired core sequences.

Core sequences *Used for preparing the building blocks for DNA nanostructures*	
Y-shaped DNA sequences	
Y1	TGGACCGATATCATATGTACATTACTATAGTACCTGAG
Y2	CTCAGGTACTATAGTAATGATCATCTATAGTACAGCCT
Y3	AGGCTGTACTATAGATGATTACATATGATATCGGTCCA
X-shaped DNA sequences	
X1	ACCGACCGATATGGTATACGGT
X2	CAGCGGTATAGGTACGGATCTG
X3	AGACGATGCGATCGCTACTACG
X4	CTCGCGGTACCTACGTACTAGC
Functional sequences *Used for introducing enzymatic function to DNA nanostructures*	
Primer sequences (append to 3' end of core sequences)	
Primer1a	AGCGTGGACACGAGTCC
Primer1b	CCAATCTTGTCACCTCTGAC
Primer2a	TGGATCCGACCGACCGATATGG
Primer2b	GAGGCTGTCTCGCGGTACC
Primer3a	ACCACAGTCCATGCCATCAC
Primer3b	TCCACCACCCTGTTGCTGTA
Single-stranded overhang sequences for ligation (append to 5' end of core sequences)	
ssOverhang1a	/5Phos/GCAA
ssOverhang1b	/5Phos/TTGC
ssOverhang2a	/5Phos/ATCC
ssOverhang2b	/5Phos/GGAT

Table 2.1: Sequences used for assembly of branched DNA structures. All sequences given in the 5' to 3' direction.

Chemical cross-linking of DNA nanostructures. Samples were cross-linked by mixing with psoralen (trioxsalen, Sigma Aldrich) at a 1:1 molar ratio between psoralen and DNA base pair, diluted in 50 mM NaCl in TE buffer to total volume of 500 μL and transferred to 24-well plate for cross-linking. Photo irradiation was performed using XL-1000 UV Crosslinker (Spectrolinker). Samples were exposed to 365 nm UV-A illumination at 2.5 mW/cm^2 for 15 minutes at room temperature. The thermostable property was confirmed with 15% Ready Gel TBE-urea polyacrylamide denaturing gel (BioRad). Samples were run at 150 V for 45 minutes at room temperature before post-staining with GelRed (Bioneer).

Branched PCR. All PCR reactions were carried out using 2x *Taq* Master Mix (New England Biolabs). PCR was carried out with branched thermostable structures replacing the standard linear primers. In a typical PCR experiment, 0.5 μM of each primer was used with 100 pg of the corresponding target. PCR products were confirmed with gel electrophoresis.

AFM imaging. PCR products were diluted in 1X TE Buffer with 2.5-10 mM magnesium chloride and deposited onto freshly cleaved mica (TED PELLA) for 90 seconds. Samples were then rinsed with deionized water and dried with

nitrogen. AFM imaging experiments were performed in air using a MultiMode 8 AFM (Bruker AXSCorporation, Santa Barbara, CA). Si tips SCANASYST-AIR (Bruker AXSCorporation, Santa Barbara, CA) with resonant frequency 45-95 kHz, spring constant 0.2-0.8 N/m, length 100-130 μm, width 20-30 μm, and tip radius < 12 nm were used in the experiments. The samples were scanned in Peak Force Tapping mode (PeakForce-Quantitative NanoMechanics, PF-QNM) at frequency 2 kHz and Z travel distance 50 nm. The AFM topography images were post-processed with Gwyddion 2.30 software. The areas containing the DNA structures were masked during post-processing to prevent any changed to the DNA contour data. The image background was flattened and leveled. Regular noise was removed with 2D FFT filtering. Areas outside the DNA contour were processed using conservative denoise and Gaussian blurring filter tools.

Preparation of PCR-generated hydrogel. Following PCR, products were concentrated using 10,000 MWCO spin filters (VWR). Resulting viscous solution was incubated at 95°C and annealed by gradual cooling from 95°C to 20°C over a duration of several hours. The resulting DNA hydrogel was stained with GelGreen dye (Bioneer) and photographed under UV illumination.

2.4 Results

Prior to carrying out PCR, we first confirmed the thermal stability of the cross-linked branch by melting curve analysis. As shown in Figure 2.4, the non-cross-linked branch exhibited a melting transition at the normal T_m of 55 °C. However, no melting transition was evident for cross-linked branches up to 95 °C, although absorbance increased slightly at high temperatures due to increased breathing within non-cross-linked regions of the double-stranded DNA. In addition, we further tested the stability of the cross-linked branch in the presence of a strong denaturant (7 M urea) in denaturing gel electrophoresis. The cross-linked branch remained intact (Figure 2.5, upper arrow), while the non-cross-linked branch was completely denatured into its constituent single-stranded DNA (Figure 2.5, lower arrows).

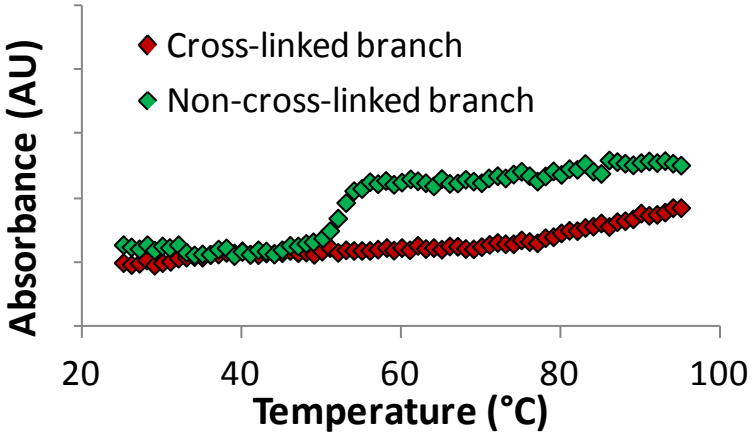


Figure 2.4: Melting curve of cross-linked branched DNA (red), and non-cross-linked branched DNA (green). Absorbance shows clear melting transition only for the non-cross-linked sample.

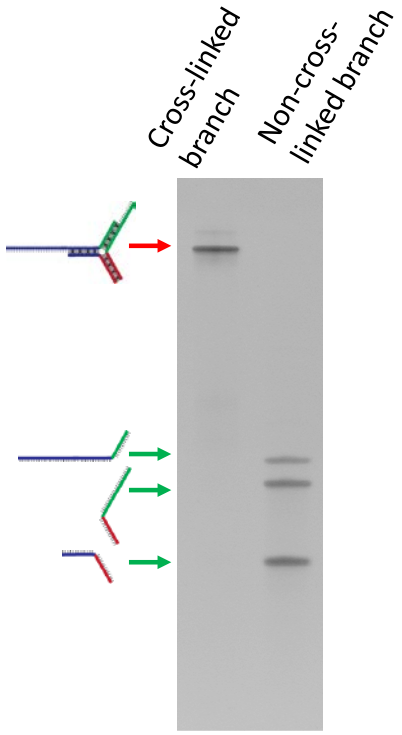


Figure 2.5: Denaturing gel electrophoresis of cross-linked branched DNA (red arrow), and non-cross-linked branched DNA (green arrows).

To further test the stability of more complex DNA nanostructures using our approach, we ligated simple Y- and X-shaped DNA together to form a variety of branched DNA nanostructures. Denaturing gel electrophoresis confirmed the stability of all nanostructures (Figure 2.6). We also tested the thermal stability of complex branched DNA nanostructures during thermal cycling (Figure 2.7).

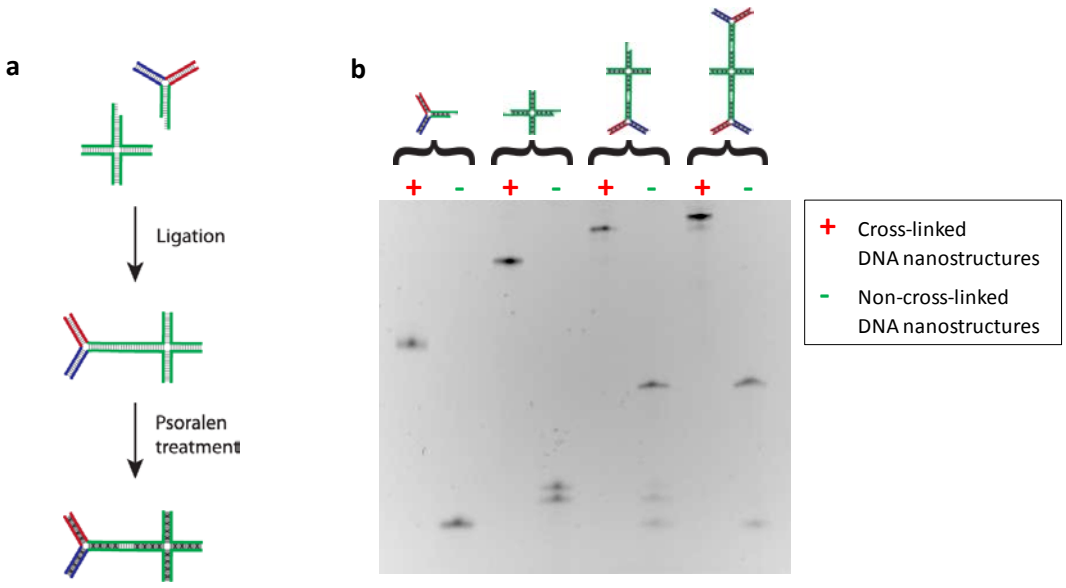


Figure 2.6: Denaturing gel electrophoresis of cross-linked branched DNA. Psoralen cross-linking can be combined with the use of DNA "building blocks" to construct a wide variety of branched DNA nanostructures. a) Illustration of modular approach to construct thermostable DNA nanostructures. After formation of Y- or X-shaped DNA "building blocks", these DNA branches are connected via DNA ligase into more complex structures. Following ligation, psoralen cross-linking may be carried out to produce thermostable DNA nanostructures. b) Denaturing gel electrophoresis of DNA nanostructures. Cross-linked nanostructures remained intact and ran at high molecular weight positions within the denaturing gel. In contrast, non-cross-linked nanostructures were denatured during electrophoresis.

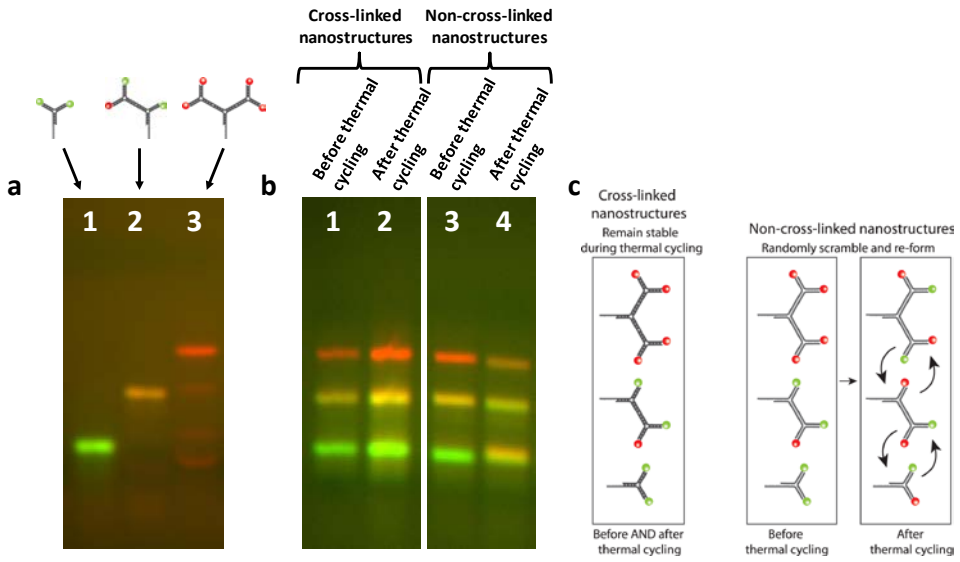


Figure 2.7: Stability of cross-linked DNA structures against thermocycling. a) Formation of three DNA nanostructures. Each DNA nanostructure had a specific size and was labelled with a specific color ratio of fluorophores. b) Equimolar mixtures of all three branched nanostructures. After thermal cycling (30 rounds of 95°C for 30 seconds, 55°C for 30 seconds, 68°C for 30 seconds), cross-linked DNA nanostructures each maintained the original color ratio, while non-cross-linked DNA nanostructures exhibited semi-random scrambling of the color ratio. This indicates that the cross-linking enabled nanostructures to be maintained even during thermal cycling. c) Schematic illustration of the behavior of DNA nanostructures during thermal cycling.

After confirming the stability of the cross-linked branched DNA, we carried out PCR replacing the conventional linear primers entirely with our modular primers. Obvious branched PCR products were observed in these samples, which confirmed that the PCR was successful and indicated that the

thermostable treatment did not interfere with the polymerase activity (Figure 2.8). We also designed more complex modular primers, which showed similar compatibility with PCR (Figure 2.9). These results demonstrated the versatility of our modular primer system, which affords the flexibility to realize a variety of branched PCR processes.

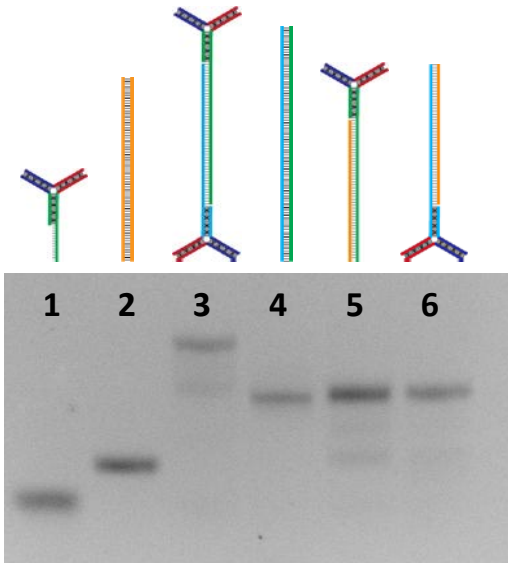
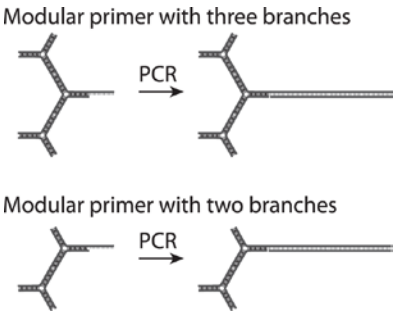


Figure 2.8: Non-denaturing gel electrophoresis of PCR products resulting from modular primers. Lane 1: Y-shaped modular primer. Lane 2: PCR product using conventional linear primers. Lane 3: PCR product using Y-shaped modular primers, resulting in branched DNA product. Lane 4: PCR product using non-cross-linked branched DNA. Branched DNA is denatured during thermal cycling, resulting in non-branched products. Lane 5: PCR product, in which only the forward linear primer was replaced with a modular primer. Lane 6: PCR product, in which only the reverse linear primer was replaced with a modular primer.

a



b

Lane	Sample
1	Template alone
2	PCR product: 2-branched primer
3	PCR product: Negative control 2-branched primer, no template
4	PCR product: Negative control 2-branched primer, no polymerase
5	PCR product: 3-branched primer
6	PCR product: Negative control 3-branched primer, no template
7	PCR product: Negative control 3-branched primer, no polymerase

Figure 2.9: Multi-branched PCR primers. a) Modular primers were formed from two Y-shaped DNA (bottom) and three Y-shaped DNA (top) ligated together. b) Gel electrophoresis of PCR products using these modular primers.

In conventional PCR, each primer can be labeled with only one fluorophore or functional moiety. In contrast, our modular primers can be labeled with multiple moieties in a controllable fashion, taking advantage of the anisotropicity of branched DNA nanostructures. In our previous work, we developed a "DNA nanobarcode" system, which used branched DNA with specific color ratios of fluorophores to correspond to specific pathogen targets.[28] Using modular primers in conjunction with the DNA nanobarcode concept, we carried out multiplexed PCR with three different target DNA sequences, as shown in Figure 2.10. In the multiplexed PCR, each PCR product was labeled with a specific color ratio. This approach enables the simultaneous amplification and labeling of target sequences in a single step.

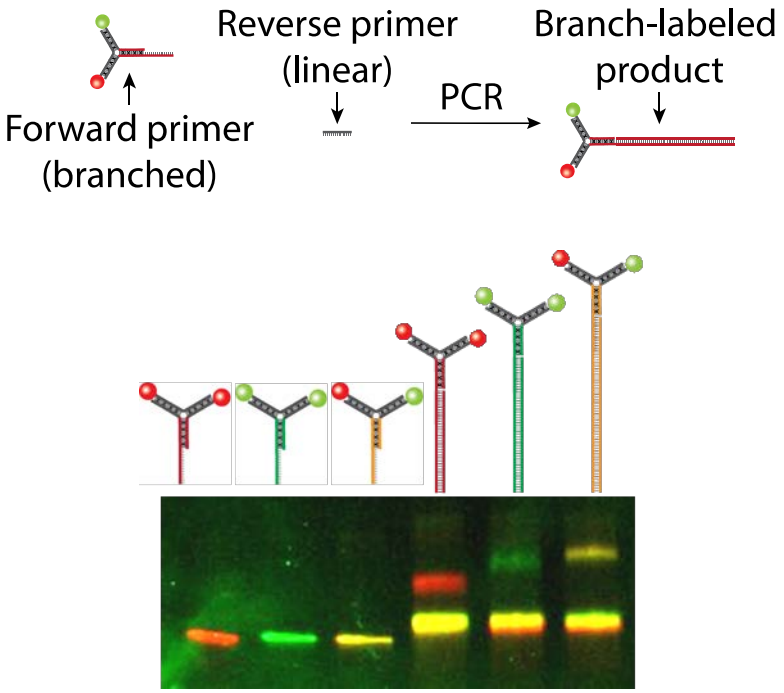


Figure 2.10: Gel electrophoresis demonstrating multiplexed PCR using multifunctional modular primers. Lanes 1-3: Three modular primers individually, showing the specific color ratio for each primer. Lanes 4-6: PCR reactions containing all primers yield PCR product with specific color ratio attached to the corresponding product.

Conventional linear primers allow polymerization to extend in only one direction, resulting in a linear product. The use of modular primers enabled polymerization in multiple directions, which resulted in multiple PCR products attached to a single branched nanostructure (Figure 2.11).

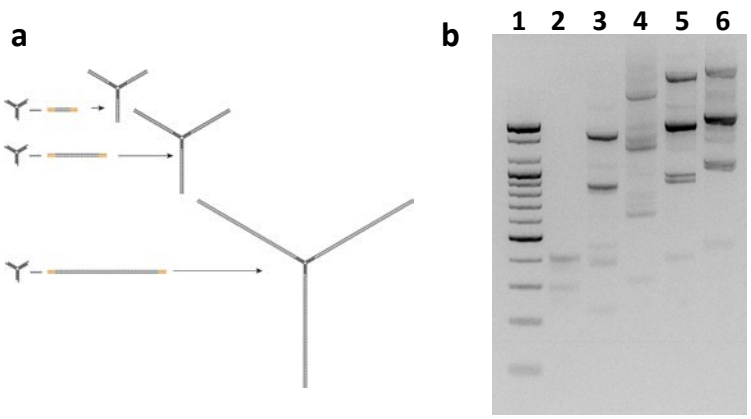


Figure 2.11: Branched PCR products. a) Illustration of Y-shaped DNA branch with three identical primers. All three primers are potentially extended during the PCR reaction, resulting in a Y-shaped PCR product. Varying the length of PCR template can give rise to different length arms on the Y-shaped PCR products. b) Electrophoresis showing extension of three identical PCR products from a single branch. Lane 1: 100bp ladder (Bioneer). Lane 2-6: PCR products using thermostable Y-shaped DNA branch with three identical primers. In each lane, a different length PCR template was used. Lanes 2 – 6 used template lengths of approximately 90, 250, 500, 750, and 1000, respectively. Distribution of PCR products was obtained, in which some Y-shaped DNA exhibited elongation of only one primer, some exhibited elongation of two primers, and some exhibited elongation of all three primers.

These branched PCR products were further confirmed by atomic force microscopy (AFM) single molecule imaging,[29] which clearly indicated that multiple products were amplified directly from a Y-shaped branch (Figure 2.12). These images also show that the lengths of PCR products can be controlled by

designing the PCR template accordingly. We further demonstrated the controlled attachment of three different PCR products on single Y-shaped branch (Figure 2.13).

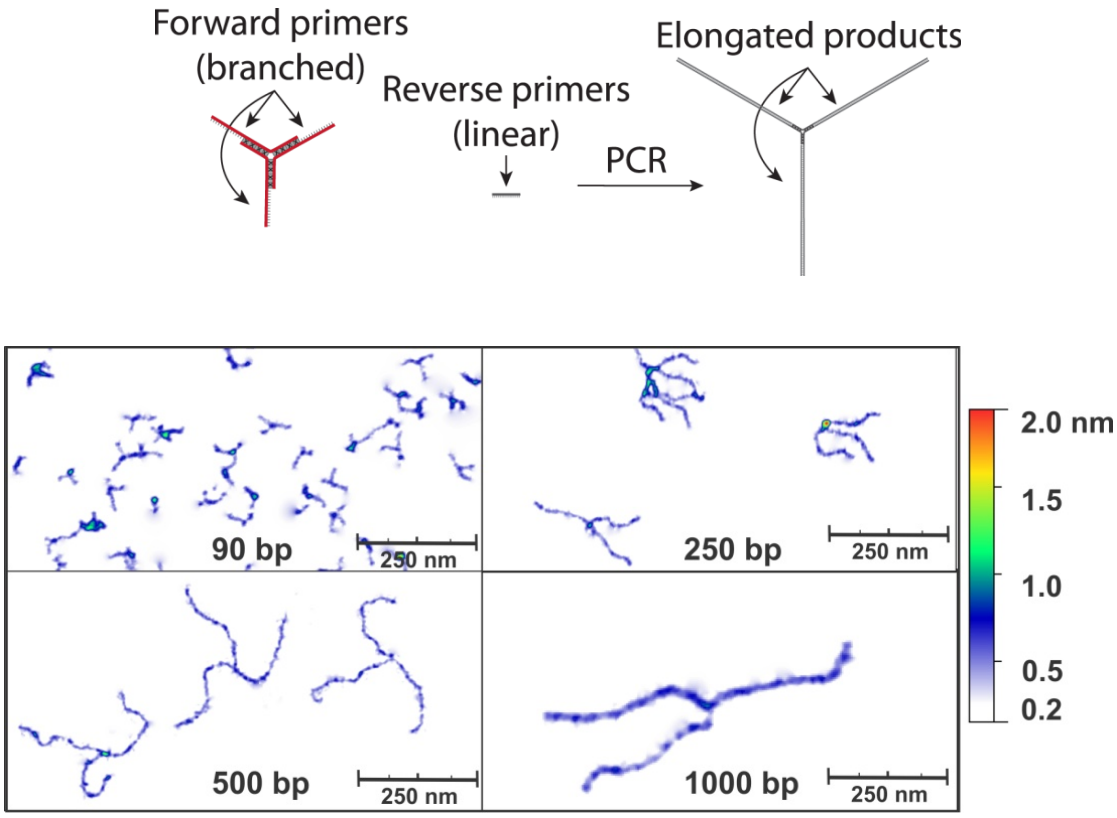


Figure 2.12: AFM of branched PCR products. Top: Illustration of starting reagents (branched forward primers and linear reverse primers) for modular primer extension via branched PCR. PCR products yield three elongated products attached to a single Y-shaped branch. Bottom: AFM images of branched structures extended via PCR. From left to right, the images show Y-shaped PCR products with 90 base pairs (bp), 250 bp, 500 bp, and 1000 bp arms, respectively. AFM topography images were post-processed with Gwyddion 2.30 software

using two-dimensional fast Fourier transform (2D FFT) filtering as well as conservative denoise and Gaussian blurring filter tools.

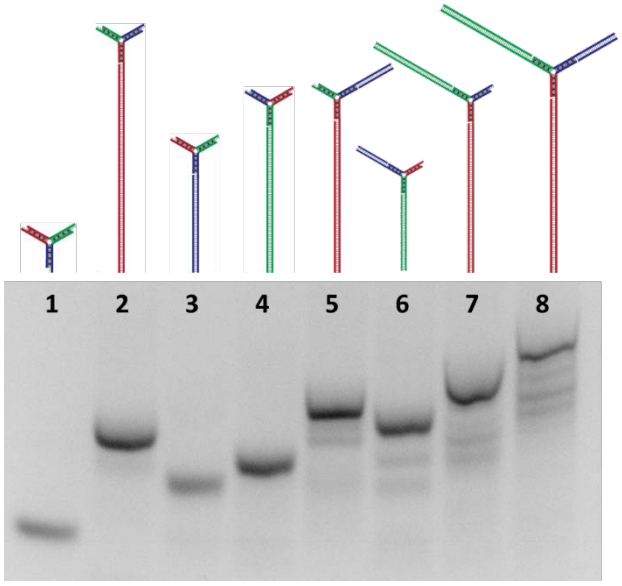


Figure 2.13: Controlled extension of branched PCR products. Gel electrophoresis shows the controlled attachment of specific PCR products, using a branched structure with three different primers. Lane 1: Y-shaped DNA branch, with three different primers. Each primer corresponds to a different PCR template. Lane 2-4: In the presence of each individual template, the corresponding primer was extended specifically. Lane 5-7: When using a combination of two templates in the same PCR, the two corresponding primers were extended specifically. Lane 8: Finally, in the presence of all three templates, all three products were amplified on a single Y-shaped branch.

Further utilizing our branched PCR, we linked multiple Y-shaped DNA branches together to form an interlinked three-dimensional network, which resulted in a bulk hydrogel. To the best of our knowledge, this is the first time a bulk hydrogel has been produced entirely from PCR processes. More specifically, we designed two modular primers: one contained three forward linear primers (red color, Figure 2.14), and the other contained three reverse linear primers (green color, Figure 2.14). During the PCR process, these primers were extended to form connections among the Y-shaped DNA branches. As a result, the PCR products formed a networked structure (Figure 2.14 and Figure 2.16), in which the Y-shaped DNA branches acted as cross-linking points within the network. We carreid out mechanical testing of this DNA hydrogel and determined the Young's modulus of approximately 1.4 kPa, as shown in Figure 2.15, which was in agreement with previous reports on DNA hydrogels and roughly equivalent to the mechanical strength of a 0.5% agarose gel.

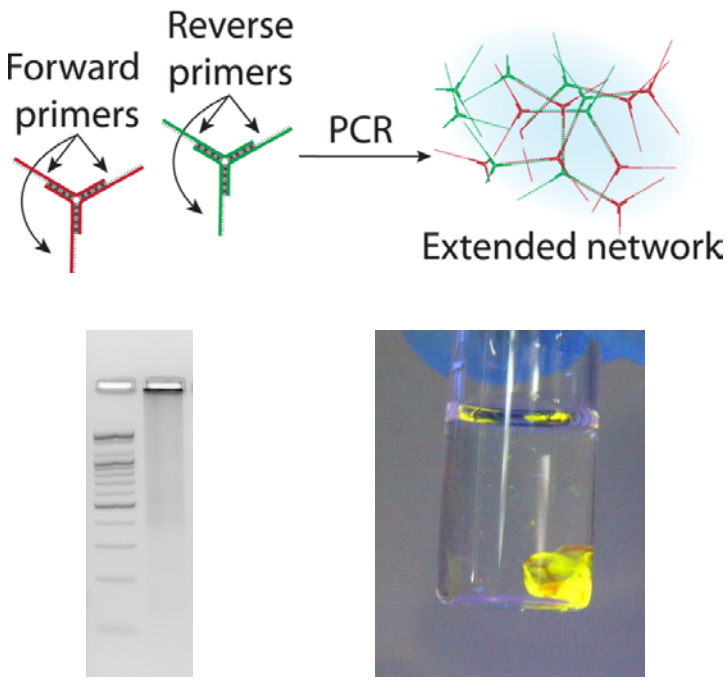


Figure 2.14: Formation of DNA hydrogel via PCR. PCR-generated hydrogel remains trapped in well during gel electrophoresis. Lane 1: 100 bp DNA ladder. Lane 2: Branched PCR product. Resulting PCR-generated hydrogel was stained with GelGreen and photographed in solution under UV illumination.

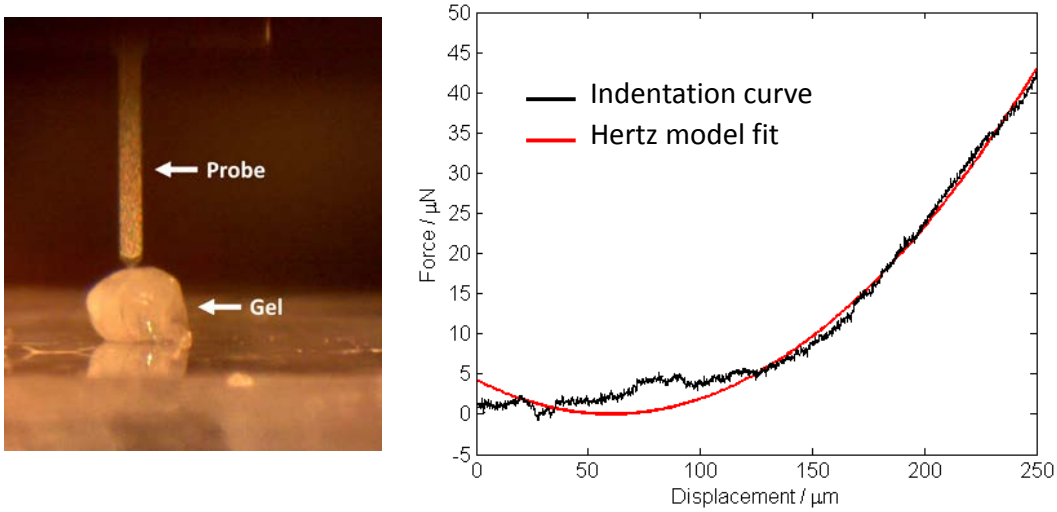


Figure 2.15: Mechanical testing of the PCR-generated hydrogel. (a) For this testing we employed a micro-mechanical analyzer (FT-S100 Microforce Sensing Probe, FemtoTools) as illustrated in the photograph. (b) We obtained an indentation curve of force per unit displacement (black line; linear region corresponds to approximately 0.3540 N/m). We applied the Hertz model treating the probe as a conical tip (D. C. Lin, E. K. Dimitriadis, F. Horkay, J Biomech Eng. 2007, 129, 430 – 440) and obtained an estimate of Young's modulus of 1.4 kPa (red line fit) which is comparable to previous works on DNA hydrogels (Y. Xing, E. Cheng, Y. Yang, P. Chen, T. Zhang, Y. Sun, Z. Yang, D. Liu, Adv. Mater. 2011, 23, 1117–21; S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach, D. Luo, Nat. Mater. 2006, 5, 797–801).

This PCR-generated hydrogel can be encoded with genetic information (by amplifying genes as the PCR template), and the resulting hydrogel could then be

utilized for cell-free protein expression. Indeed, substantial production of a reporter protein, green fluorescent protein (GFP), was observed within the gel after we incorporated GFP gene into the PCR with modular primers and carried out *in vitro* expression (Figure 2.17). This approach is analogous to our previous work on cell-free expression with DNA hydrogels, but uses PCR rather than ligation as the mechanism for introducing genes into the gel.[29], [30] This confirmed that branched PCR with modular primers can create a generic structure while simulateously incorporating the genetic functions of the resulting PCR products.

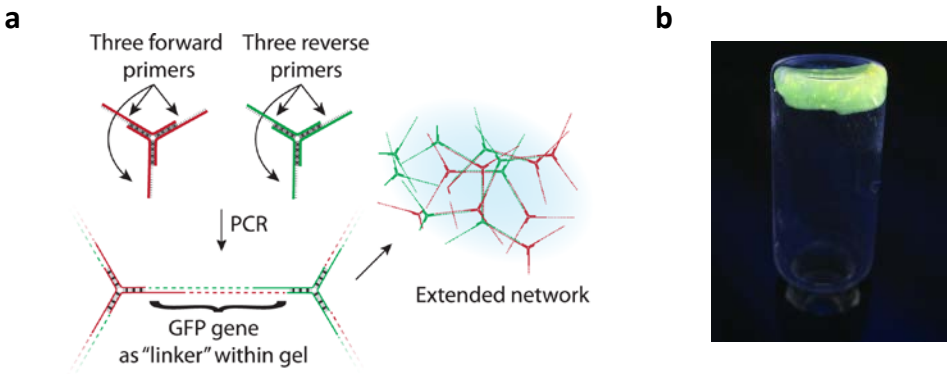


Figure 2.16: PCR-generated DNA hydrogel containing gene. Modular primers were prepared to form an aggregate structure using GFP gene as a template. (a) All three arms of the Y-shaped modular primer could be extended during the

PCR process, resulting in a highly interconnected extended network structure. b) Photograph of bulk scale PCR-generated DNA hydrogel with excess solution removed in an inverted vial under UV illumination. The green color in this image is due to fluorescence from DNA-specific dye (GelGreen).

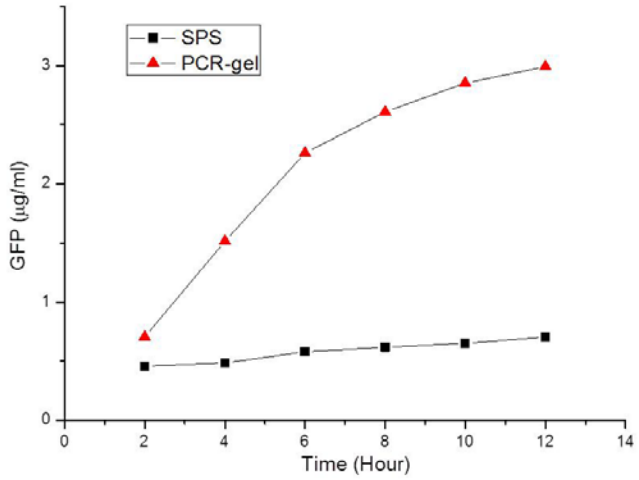


Figure 2.17: Cell free expression using PCR-generated DNA hydrogel. Modular primers were prepared to form a hydrogel using GFP gene as a template. The resulting PCR-generated DNA hydrogel was used for cell-free protein production. The resulting expression indicated enhancement of protein production from the PCR-generated hydrogel in comparison with a solution phase system (SPS).

2.5 Conclusion

In conclusion, we have developed thermostable branched DNA nanostructures. These structures can be utilized as modular primers for branched PCR, allowing

us to take advantage of both the self-assembly and biological capabilities of DNA in a single PCR process. This branched PCR enabled the attachment of multiple moieties, such as fluorescence dyes and genes, onto a single branched PCR product, as well as the production of a PCR-generated DNA hydrogel. We expect this modular primer system to be further expanded to utilize branched PCR with more complicated DNA-based nanoarchitectures, such as DNA origami, DNA canvas, or molecular devices,[6], [7] and we envision that thermostable DNA nanostructures will have great utility for DNA-based materials in real world applications.

2.6 References

[1] N. C. Seeman, "DNA in a material world.," *Nature*, vol. 421, no. 6921, pp. 427–31, Jan. 2003.

[2] U. Feldkamp and C. M. Niemeyer, "Rational design of DNA nanoarchitectures.," *Angewandte Chemie International Edition*, vol. 45, no. 12, pp. 1856–76, Mar. 2006.

[3] J. Bath and A. J. Turberfield, "DNA nanomachines.," *Nature Nanotechnology*, vol. 2, no. 5, pp. 275–84, May 2007.

[4] A. V. Pinheiro, D. Han, W. M. Shih, and H. Yan, "Challenges and opportunities for structural DNA nanotechnology," *Nature Nanotechnology*, vol. 6, no. 12, pp. 763–772, Nov. 2011.

[5] S. Peng, T. L. Derrien, J. Cui, C. Xu, and D. Luo, "From Cells to DNA materials," *Materials Today*, vol. 15, no. 5, pp. 190–194, 2012.

[6] P. W. K. Rothemund, "Folding DNA to create nanoscale shapes and patterns.," *Nature*, vol. 440, no. 7082, pp. 297–302, Mar. 2006.

[7] B. Wei, M. Dai, and P. Yin, "Complex shapes self-assembled from single-stranded DNA tiles.," *Nature*, vol. 485, no. 7400, pp. 623–6, May 2012.

[8] C. Zhang, C. Tian, F. Guo, Z. Liu, W. Jiang, and C. Mao, "DNA-directed three-dimensional protein organization.," *Angewandte Chemie International Edition*, vol. 51, no. 14, pp. 3382–5, Apr. 2012.

[9] S. Hamada and S. Murata, "Substrate-assisted assembly of interconnected single-duplex DNA nanostructures.," *Angewandte Chemie International Edition*, vol. 48, no. 37, pp. 6820–3, Jan. 2009.

[10] C. G. Evans, R. F. Hariadi, and E. Winfree, "Direct atomic force microscopy observation of DNA tile crystal growth at the single-molecule level.," *J Am Chem Soc*, vol. 134, no. 25, pp. 10485–92, Jun. 2012.

[11] Y. Fu, D. Zeng, J. Chao, Y. Jin, Z. Zhang, H. Liu, D. Li, H. Ma, Q. Huang, K. V Gothelf, and C. Fan, "Single-step rapid assembly of DNA origami nanostructures for addressable nanoscale bioreactors.," *J Am Chem Soc*, vol. 135, no. 2, pp. 696–702, Jan. 2013.

[12] C. Lin, R. Jungmann, A. M. Leifer, C. Li, D. Levner, G. M. Church, W. M. Shih, and P. Yin, "Submicrometre geometrically encoded fluorescent barcodes self-assembled from DNA.," *Nature Chemistry*, vol. 4, no. 10, pp. 832–9, Oct. 2012.

[13] C. M. Niemeyer, M. Adler, and R. Wacker, "Detecting antigens by quantitative immuno-PCR.," *Nature Protocols*, vol. 2, no. 8, pp. 1918–30, Jan. 2007.

[14] B. Li, X. Chen, and A. D. Ellington, "Adapting enzyme-free DNA circuits to the detection of loop-mediated isothermal amplification reactions.," *Analytical Chemistry*, vol. 84, no. 19, pp. 8371–7, Oct. 2012.

[15] L.-L. Li, P. Wu, K. Hwang, and Y. Lu, "An Exceptionally Simple Strategy for DNA-Functionalized Up-Conversion Nanoparticles as Biocompatible Agents for Nanoassembly, DNA Delivery, and Imaging.," *J Am Chem Soc*, vol. 135, no. 7, pp. 2411–4, Feb. 2013.

[16] C. K. McLaughlin, G. D. Hamblin, K. D. Hänni, J. W. Conway, M. K. Nayak, K. M. M. Carneiro, H. S. Bazzi, and H. F. Sleiman, "Three-dimensional organization of block copolymers on 'DNA-minimal' scaffolds.," *J Am Chem Soc*, vol. 134, no. 9, pp. 4280–6, Mar. 2012.

[17] Y. Xing, E. Cheng, Y. Yang, P. Chen, T. Zhang, Y. Sun, Z. Yang, and D. Liu, "Self-assembled DNA hydrogels with designable thermal and enzymatic responsiveness.," *Advanced Materials*, vol. 23, no. 9, pp. 1117–21, Mar. 2011.

[18] S. H. Um, J. B. Lee, N. Park, S. Y. Kwon, C. C. Umbach, and D. Luo, "Enzyme-catalysed assembly of DNA hydrogel.," *Nature Materials*, vol. 5, no. 10, pp. 797–801, Oct. 2006.

[19] J. B. Lee, S. Peng, D. Yang, Y. H. Roh, H. Funabashi, N. Park, E. J. Rice, L. Chen, R. Long, M. Wu, and D. Luo, "A mechanical metamaterial made from a DNA hydrogel.," *Nature Nanotechnology*, vol. 7, no. 12, pp. 816–20, Dec. 2012.

[20] G. D. Cimino, H. B. Gamper, S. T. Isaacs, and J. E. Hearst, "Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry.," *Annual Review of Biochemistry*, vol. 54, pp. 1151–93, Jan. 1985.

[21] M. Tagawa, K. Shohda, K. Fujimoto, and A. Suyama, "Stabilization of DNA nanostructures by photo-cross-linking," *Soft Matter*, vol. 7, no. 22, p. 10931, 2011.

[22] A. Rajendran, M. Endo, Y. Katsuda, K. Hidaka, and H. Sugiyama, "Photo-cross-linking-assisted thermal stability of DNA origami structures and its application for higher-temperature self-assembly.," *J Am Chem Soc*, vol. 133, no. 37, pp. 14488–91, Sep. 2011.

[23] J. K. Lee, Y. H. Jung, J. B.-H. Tok, and Z. Bao, "Syntheses of organic molecule-DNA hybrid structures.," *ACS Nano*, vol. 5, no. 3, pp. 2067–74, Mar. 2011.

[24] S. Keller, J. Wang, M. Chandra, R. Berger, and A. Marx, "DNA polymerase-catalyzed DNA network growth.," *J Am Chem Soc*, vol. 130, no. 40, pp. 13188–9, Oct. 2008.

[25] J. K. Lee, Y. H. Jung, R. M. Stoltenberg, J. B.-H. Tok, and Z. Bao, "Synthesis of DNA-organic molecule-DNA triblock oligomers using the amide coupling reaction and their enzymatic amplification.," *J Am Chem Soc*, vol. 130, no. 39, pp. 12854–5, Oct. 2008.

[26] Y. Li, Y. D. Tseng, S. Y. Kwon, L. D'Espaux, J. S. Bunch, P. L. McEuen, and D. Luo, "Controlled assembly of dendrimer-like DNA.," *Nature Materials*, vol. 3, no. 1, pp. 38–42, Jan. 2004.

[27] J. B. Lee, Y. H. Roh, S. H. Um, H. Funabashi, W. Cheng, J. J. Cha, P. Kiatwuthinon, D. A. Muller, and D. Luo, "Multifunctional nanoarchitectures from DNA-based ABC monomers.," *Nature Nanotechnology*, vol. 4, no. 7, pp. 430–6, Jul. 2009.

[28] Y. Li, Y. T. H. Cu, and D. Luo, "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes.," *Nature Biotechnology*, vol. 23, no. 7, pp. 885–9, Jul. 2005.

[29] N. Park, S. H. Um, H. Funabashi, J. Xu, and D. Luo, "A cell-free protein-producing gel.," *Nature Materials*, vol. 8, no. 5, pp. 432–7, May 2009.

[30] N. Park, J. S. Kahn, E. J. Rice, M. R. Hartman, H. Funabashi, J. Xu, S. H. Um, and D. Luo, "High-yield cell-free protein production from P-gel.," *Nature Protocols*, vol. 4, no. 12, pp. 1759–70, Jan. 2009.

CHAPTER 3

POINT-OF-CARE NUCLEIC ACID DETECTION

3.1 Challenge of point-of-care diagnostics

Recent developments in nanotechnology have led to significant advancements in point-of-care (POC) nucleic acid diagnostics. The ability to sense DNA and RNA in a portable format will play a key role in personalized medicine with important applications for a range of clinical and non-clinical settings in both the developing and developed world. In this chapter, nanotechnology-driven innovations are discussed in the context of three key process components for nucleic acid detection: sample preparation, target amplification, and detection/read-out modalities. The advancements realized by nanotechnology are making POC nucleic acid detection increasingly prevalent, and revolutionizing diagnosis to shift toward more personalized and decentralized testing.

3.2 Introduction and background for point-of-care (POC) detection

Point-of-care (POC) diagnosis, testing carried out at or near the site of the patient, is an extremely attractive concept for expanding the access and efficiency of diagnostic testing beyond hospitals and centralized laboratory-based testing. POC detection can be particularly valuable for developing world or resource-limited settings.[1–3] In the developing world context, portable diagnosis could offer increased access to tests for infectious diseases such as HIV, malaria, tuberculosis, and sexually transmitted infections.[4] In addition, POC detection is of growing importance for the developed world, for contexts such as at-home care and minimally complex and equipment-free tests that could someday be offered at the drug store or basic clinic.[5–8] Furthermore, the technology developed for POC detection covers a broad range of applications, as represented in Figure 3.1, including testing in agriculture (crops and food supply), veterinary medicine and animal health (pets and livestock), and in the environment (air, soil, and water contamination).[1], [9], [10]

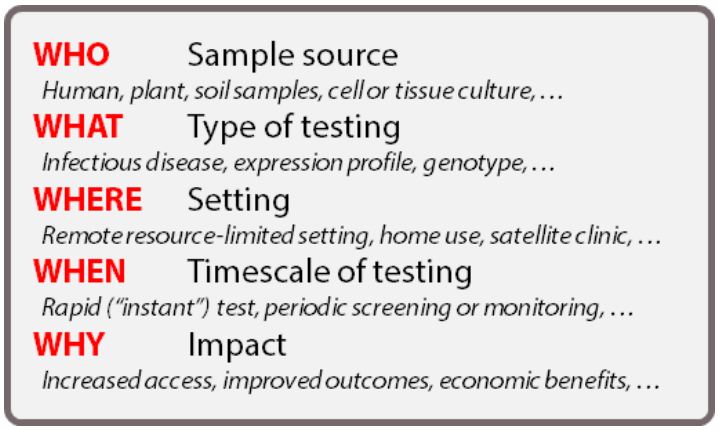


Figure 3.1: The "5 W's" regarding POC diagnosis of nucleic acids. With applications extending well beyond the limits of conventional hospital and laboratory-based testing, the potential scope of POC detection is remarkably broad. The "how" of POC detection, absent from this figure, constitutes the main subject of this chapter.

POC detection for nucleic acids is of particular interest given the extreme importance of DNA and RNA as diagnostic targets with applications ranging from genotyping and genetic prognostics, to expression profiling and detection of infectious disease.[11–15] In current clinical practice, nucleic acid diagnostics are widely used for situations involving infectious disease and cancer (e.g. tumor genotyping).[16], [17] Nucleic acid-based testing is a popular and preferred method for monitoring epidemics and outbreaks of new diseases (e.g. H7N9).[18], [19] Compared to immunological biomarkers, nucleic acids have several important practical advantages, including: (1) straightforward design of

specific recognition elements (primers and probes) with predictable behaviors and binding affinities, and (2) straightforward compatibility with enzymatic target amplification methods such as PCR, which enable nucleic acid detection to be extremely sensitive.

POC detection represents a complex engineering challenge that will benefit from nanotechnology-enabled functionalities and capabilities, as shown in Figure 3.2. For example, metal nanoparticles (e.g. gold nanoparticles) have been widely used for biosensing applications,[20], [21] due to their unique characteristics such as their high degree of polyvalency and plasmonic properties.[22] As another example, DNA-based nanostructures have been designed in a controlled fashion to form nanoscale shapes and patterns, which interfaced directly with target nucleic acids for detection applications.[23–25] In addition, DNA nanotechnology can be utilized in the creation of hybridization-based circuits and reaction networks and DNAzymes and RNAzymes for non-enzymatic target and signal amplification.[26] Other engineered nanomaterials such as carbon nanotubes and nanowires have been utilized for highly sensitive optical and electrical sensing.[27] Quantum dots and other nanomaterials have been widely used as superior labels and contrast agents for fluorescent detection and diagnostic imaging[28–30] as wells as for detection of proteins and small molecules.[20], [31], [32] In general, nanoscale materials have higher surface-to-

volume ratios, associated with improved catalytic and binding properties, which can facilitate enhanced transduction of target-binding events into signals.[33], [34] This has been utilized for improved binding and transport properties in nanoporous materials such as hydrogels and polymeric and metal/metal-oxide solid supports.[35–39] Overall, nanotechnology is poised to play a key role in the development of next generation POC technologies.[40–43]

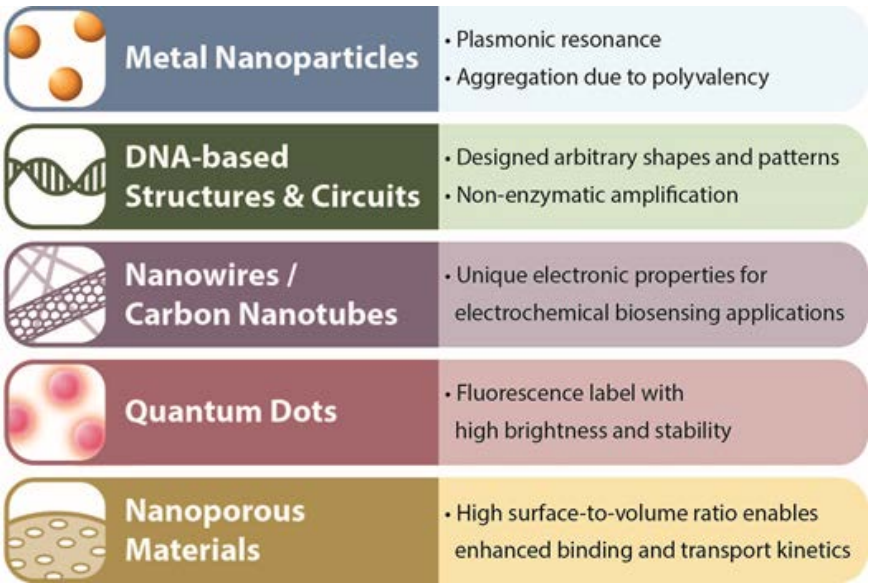


Figure 3.2: A sampling of nanotechnology concepts and nanomaterials that have been utilized for POC nucleic acid detection.

The ideal portable detection method will meet all the requirements of traditional centralized laboratory-based testing, while also overcoming the additional

challenges associated with POC testing, such as uncontrolled environmental conditions (temperature and humidity), inconsistent or non-existent electrical power supply, non-sterile conditions, and operation by untrained or minimally trained personnel. The attributes generally required for POC detection are illustrated by the ASSURED criteria, as represented in Figure 3.3.[3] These criteria emphasize the range of priorities required for consideration in the development of innovative and efficient POC methods and devices. Whenever possible, POC devices should involve low cost materials, minimally complex procedures and equipment requirements, and reliable performance even under less-than-ideal operating conditions. Devices that do not satisfy the ASSURED criteria may be poorly-suited for implementation in the field. This review highlights a representative sampling of the diverse innovations that have been developed to satisfy the POC ASSURED criteria, which can act as component technologies for design of the ideal POC detection device.

Figure 3.3: The ASSURED criteria for POC detection, as originally developed by the World Health Organization. We note that these criteria are not meant to be limiting, and viable technical options outside the scope of these guidelines may still be worth pursuing.

We structure this review to reflect the three distinct operational steps that are typically required for POC detection: sample preparation and processing, target amplification, and detection and read-out (Figure 3.4). Although each step can be considered individually, it is important to emphasize that the key challenge for development of POC nucleic acid diagnostic methods is the integration of all these key steps into a unified process workflow and preferably within a single device.

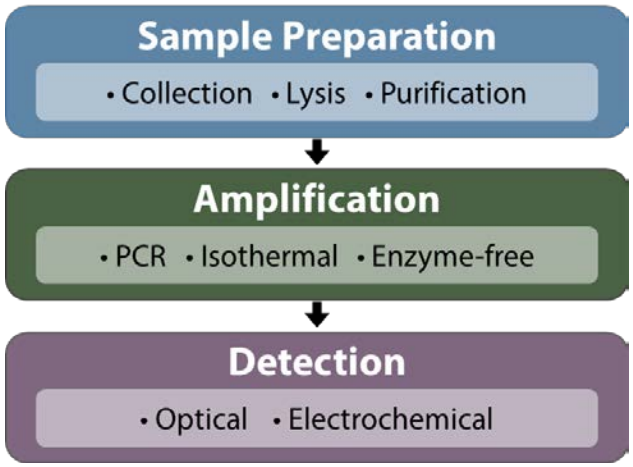


Figure 3.4: The outline of this review is based on the three distinct operational steps that are typically required for POC detection: sample preparation and processing, target amplification, and signal detection and read-out. The ideal POC nucleic acid diagnostic device will smoothly integrate these key steps into a single process.

3.3 Sample Preparation

Sample preparation has been recognized as one of the most critical considerations for effective nucleic acid POC diagnostics.[44–49] The primary challenge of sample preparation is the diversity of sample types. Test specimens may be collected from a broad range of sources, including: human or animal (e.g. body fluids, swabs, or tissue samples), plant, cell or virus culture, and environmental samples (e.g. water, soil, or air). The samples of interest may also have a diverse range of physical and chemical characteristics (e.g. viscosity, pH,

salinity), requiring a variety of approaches for sample collection and pretreatment. For example, in some specimens (e.g. stool or soil), most of the sample may consist of solid content, thus requiring additional sample dilution and pretreatment.

In most cases, sample preparation involves lysis in order to disrupt the target cells (e.g. pathogenic virus or bacteria) and release nucleic acid material.[50] Lysis is most commonly carried out through chemical means using denaturants, but may also be accomplished through freeze-thawing, sonication, heating, and other methods. Near infrared heating of gold nanorods has been utilized to achieve one step DNA extraction and real-time PCR of pathogens in a single chamber.[51] It is also important to note that in some detection scenarios, lysis is unnecessary; for example, certain nucleic acids associated with cancer can circulate in the blood stream, and cell-free nucleic acids may also be present in the urine.[52–54]

Following sample lysis, nucleic acid material is typically purified from the remainder of the sample. Purification is required for consistent performance of downstream amplification and detection read-out steps. In particular, untreated samples frequently contain inhibitors that interfere with PCR or other target amplification processes.[55] Purification is also an opportunity for target

enrichment, which is beneficial because nucleic acid concentrations in native samples may be insufficient for sensitive detection. Through enrichment, the concentration of nucleic acids can be increased and milliliter sample volumes can often be reduced to tens of microliters. Sample purification is also important for maintaining the integrity of the target nucleic acid by removing or inhibiting nucleases; this is particularly important for RNA, which is easily degraded in complex media. By far the most common method for nucleic acid purification and enrichment is the Boom method, due to its convenience and effectiveness to obtain highly pure product.[56] This is a solid phase extraction (SPE) method in which (1) the test sample is mixed with a chaotropic agent that simultaneously denatures proteins and facilitates nucleic acid binding to a solid phase (usually silica), (2) the bound nucleic acid is washed to remove components of the original sample other than nucleic acid, and (3) the nucleic acid is eluted at a higher concentration in sterile buffer.

To carry out the Boom method, one common approach is to pass lysed samples and wash buffers over the solid phase via some actuation force. This approach is currently used in a large number of nucleic acid purification devices, in which the actuation force can be pressure,[57–59] capillary force,[60], [61] or centrifugal force.[62], [63] There is an increased advantage in the use of micro- and nano-structured materials, which can offer higher surface areas for increased binding

of nucleic acids.[64] For example, a nanoporous monolithic aluminum oxide membrane (AOM) was used for DNA extraction and real-time polymerase chain reaction (RT-PCR) amplification of the extracted DNA.[65] In this work, lysed samples were filtered through the nanoporous AOM to extract the DNA. PCR reagents were added to each of the wells and the chip was thermocycled directly on the AOM membrane.

Another strategy for purification uses magnetic microparticles to capture nucleic acids. For example, iron core microparticles coated with a nanometer layer of silica are commercially available.[66] After binding to nucleic acids, these beads can be magnetically trapped as wash and elution buffers are passed over the bead surface. The use of a magnet is extremely compatible for POC applications. For example, Duarte and coworkers demonstrated a sample preparation device that used external magnets to generate movement of magnetic beads through a micro-chamber, ensuring thorough sample mixing and increasing interaction with the beads.[67] This approach has also been used with pinned immiscible interfaces in microchannels, for which either air/liquid or oil/aqueous interfaces can be used. These interfaces act as barriers, separating the lysis and elution chambers and preventing mixing of these two solutions.[68–71] This method streamlines the extraction process, replacing all washing steps with a single traverse of the immiscible barrier.

3.4 Target Amplification Strategies

Once sample preparation (process step one, as in Figure 3.4) is completed, nucleic acid detection often requires some form of target amplification (process step two) in order to obtain clinically relevant sensitivity from small amounts of samples.[72–74] This target amplification step introduces substantial operational complexity to the nucleic acid detection process, and is therefore undesirable. If the target amplification process is eliminated altogether this may be referred to as "direct detection" of nucleic acids. Direct detection methods greatly simplify the diagnostic procedure but require extremely sensitive read-out to accurately detect the non-amplified nucleic acid target. Direct detection of nucleic acids must overcome fundamental problems including (1) low signal requiring extremely sensitive signal amplification, and (2) low target concentrations leading to slow reaction kinetics. Thus, despite the added process complexity associated with target amplification, most nucleic acid detection strategies are well-justified in their use of target amplification in order to overcome these problems.

3.4.1 Enzyme-based target amplification: PCR and isothermal amplification methods

This target amplification process usually involves an enzymatic reaction such as polymerase chain reaction. Due to the popularity of PCR as a target amplification technique in clinical and laboratory settings, PCR has been widely adapted and implemented in POC detection as well.[75], [76] In the commercial context, Cephied's GeneXpert is an FDA-approved benchtop system that utilizes PCR and enjoys widespread use.[77] Taking advantage of the microfluidic format that is widely used for POC diagnostics, significant work has been done to increase the speed of portable PCR, which could potentially offer rapid results to patients.[78] Using these and other innovations, PCR-based POC methods have demonstrated great success.[65], [79] Nevertheless, the thermal cycling required by PCR (necessitating heaters, temperature sensors, and increased process and device complexity) represents an obstacle for POC. Thus, a substantial amount of work has focused on the development of techniques that avoid the thermal cycling requirement. I will briefly discuss non-PCR target amplification methods including isothermal amplification and non-enzyme amplification, because these methods are more amenable to POC detection. In particular, I will describe the use of nanotechnology innovations (e.g. DNA-based reaction networks that has developed from DNA nanotechnology) for non-enzymatic target amplification as

a particular area of interest.

Unlike PCR, isothermal amplification methods are designed so that amplification occurs at one constant temperature. This modification translates into fewer equipment requirements, making isothermal amplification ideal for integration into microfluidic devices for POC applications. Several different isothermal amplification methods are available, and I will briefly address some of the most popular, including LAMP, NASBA, RCA, and HDA (Figure 3.5).[74]

Loop mediated isothermal amplification (LAMP) is an increasingly popular technique for POC applications; it has the advantage of being performed at a single temperature with only one type of enzyme. The LAMP procedure uses four primers and polymerase with strand displacement activity to achieve a cyclical amplification process based on spontaneous formation of stem-loop DNA structures.[80] The LAMP method has a reputation for being rapid (<1 hour) and has a relatively broad working range (60-65˚C) which reduces the need for highly stable heating devices, making it an attractive method for POC diagnostics. As a result there are many examples of various LAMP assays which have been developed for resource poor areas.[81–84] Extent of the reaction can be determined by measuring the turbidity of the reaction solution or via an intercalating DNA dye like SYBR GREEN I.[85] One challenge of the LAMP

method is false positive results deriving from accidental amplification of non-target DNA sequences. To overcome this drawback, researchers have incorporated sequence-specific fluorescent probes such as molecular beacons to add an additional layer of specificity.[86]

Nucleic acid sequence based amplification (NASBA)[87] is a well-established isothermal amplification technique that offers excellent amplification of RNA sequences, producing single-stranded RNA products. Compared to other target amplification methods, NASBA is a popular choice because it can detect single-stranded RNA, and because it generates single-stranded RNA products that allow for convenient hybridization in downstream detection steps. Using a mixture of reverse transcriptase, primers, RNase H, and T7 RNA polymerase, NASBA amplifies single stranded RNA sequences by cyclically (1) forming DNA:RNA hybrids with reverse transcriptase, (2) degrading the RNA with RNase H, (3) creating dsDNA using with reverse transcriptase, and (4) and generating many copies of single-stranded RNA with RNA polymerase. NASBA requires three different enzymes (reverse transcriptase, RNAse H, and RNA polymerase), which may increase procedural complexity. Zhao et al, used NASBA to create is a low cost and potentially disposable device which can detect down to 64 cells in a liter of solution.[88] NASBA has also been used for the simultaneous amplification of mRNA and miRNA, which may make it an

excellent process for screening for indicators of cancer.[89]

Rolling circle amplification (RCA) utilizes a DNA polymerase with strand-displacement activity to continuously elongates a hybridized primer from a circular DNA template.[90], [91] This process results in long DNA repeats which can be labeled with DNA-binding dyes. The most distinctive aspect of RCA is the requirement for a circular template for amplification, which makes RCA uniquely well suited for amplification of naturally circular DNA molecules such as plasmids and certain virus genomes. RCA can also detect linear DNA, but requires an additional circularization step; this step increases specificity but increases process complexity. Alternately, the target nucleic acid can be utilized as a primer to initiate the RCA process. To increase sensitivity, repeated hairpin formation within the RCA products has been used to increase signal by forming dsDNA which has a high affinity for DNA dyes like SYBR green [92]. To further increase the speed of target amplification, additional primers can be utilized to bind to the repeating sequences in the RCA product (called branching RCA, or geometrical RCA), triggering additional amplification to improve the performance of POC detection. This can yield 10^9 or more copies of the template in under an hour and has been shown to detect as few as ten molecules.[93] In another strategy, RCA was used to detect miRNA to generate a single-stranded product containing DNAzyme, giving rise to a color signal.[94]

Helicase dependent amplification (HDA) mimics nature by utilizing helicase to separate double stranded DNA.[95] Thus, HDA is uniquely well-suited for detection of double-stranded DNA targets since it does not require any initial heat denaturation step. In many cases, HDA has exhibited a detection limit ranging from 10 to 10^3 DNA copies in under an hour.[96–99] A notable example of a HDA based system was produced by Kivlehan et al. in which real-time electrochemical monitoring was enabled using an intercalating redox probe.[100] Mahalanabis and coworkers demonstrated HDA in a device that integrated bacterial lysis, nucleic acid extraction, and detection with a fluorescent reporter, achieving detection of down to ten colony forming units of *E. coli*.[59] In another example of HDA-based detection for POC, Huang et al achieved complex sample preparation and DNA amplification using only simple materials and no external electrical power.[58]

Aside from the isothermal amplification methods highlighted above, a wide variety of additional methods have also been reported, including transcription-mediated amplification (TMA),[101] strand displacement amplification (SDA),[102] multiple strand displacement (MDA, often used for nonspecific amplification of genomic DNA),[103] recombinase polymerase amplification (RPA),[104] and cross priming amplification (CPA)[105], among many others.[106] This growing collection of isothermal amplification techniques may

serve as a library of candidates for incorporation into any potential POC nucleic acid detection device.

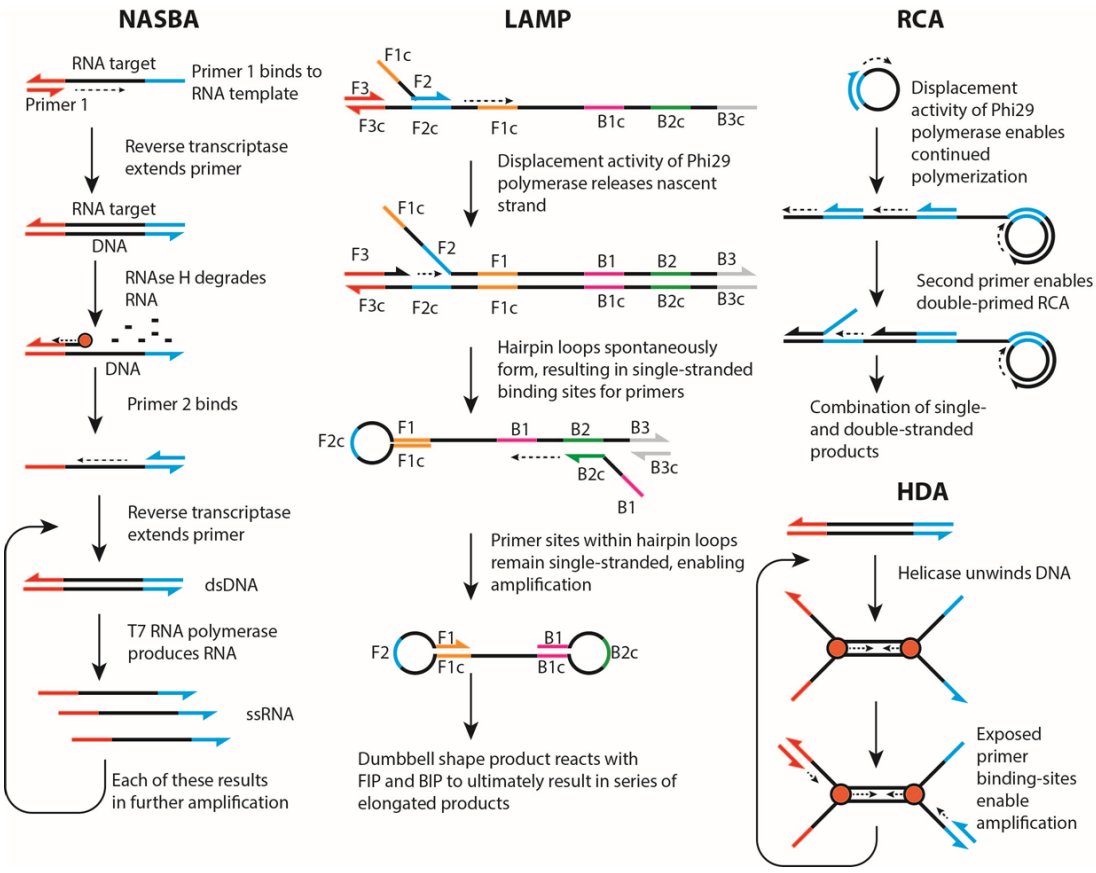


Figure 3.5: Schematics of several of the most popular isothermal target amplification methods for nucleic acid amplification. NASBA is well-established for amplification of RNA targets. LAMP requires only one single enzyme (most isothermal amplification methods require at least two). RCA works best for detection of circular and single-stranded DNA targets. HDA, inspired by nature's method for DNA replication, uses helicase to unwind DNA.

3.4.2 Non-enzymatic target amplification

Although enzymes, particularly polymerases, are effective for target amplification of nucleic acids, they introduce certain challenges for POC detection. Specifically, enzymes are often unstable at POC settings because they require special conditions including refrigeration for transport, storage, and handling. Because of these drawbacks, enzyme-free methods have attracted a great deal of attention for POC detection.

Current enzyme-free methods are mostly motivated by academic interest and carried out in laboratory settings and not POC; however, the technology is promising for POC applications due to the predictability of reactions that are mainly based on hybridization of nucleic acids. Nevertheless, these non-enzymatic technologies represent one important area where nanotechnology (particularly DNA and RNA nanotechnology) can play a critical role in the development of novel POC diagnostics. In particular, we focus on DNA hybridization-based methods as the amplification mechanism.

The basic mechanism for achieving DNA-based chain reactions using strand displacement concepts was introduced by Yurke *et al.*[107] Through strand displacement, a partially hybridized strand is exchanged into a fully hybridized strand using a single-stranded region (toehold) as a starting point. Seelig and

coworkers improved upon this idea by creating a metastable DNA fuel complex, which increased the stability of the fuel strands about two orders of magnitude.[108] This design was later adapted to the input signal amplifier of DNA logic gates.[109] Recently, large-scale DNA logic gates with signal amplification were realized by Qian and Winfree.[110] In order to obtain more rapid signal amplification, autocatalysis has been used to attain an acceleration of signal amplification, through which a repetitive reaction increased the number of catalysts.[111] These implementations show the robustness of DNA-based logic gates and amplification techniques even in large scales, which can be used as a strong and reliable methodology for future POC diagnostics.

Hairpin motifs are also widely used in this field. One approach towards amplification using this motif is to produce long output double strands triggered by inputs, instead of the increase in concentration. Hybridization chain reaction (HCR) achieves this using single stranded DNA as a trigger to produce long double-stranded DNA.[112] This system was also implemented *in situ*, using it as an imaging technique for mRNA expression in zebrafish embryos.[113] This example, which succeeded in practical environment, clearly indicates the amplification of the signal (and also detection) is also possible for POC as well. Yin and colleagues used the hairpin motif to design diverse types of reactions based on simple but powerful methodology.[114] Moreover, Li *et al.* reported

several hairpin-based reactions that including amplification and also showed that the reaction could be used for the detection of RNA and small molecules in a modular fashion.[115] These series of examples demonstrated the potential of the hairpin-based motifs towards actual use in POC diagnostics in terms of robustness and modularity.

Isothermal amplification strategies can be used in conjunction with non-enzymatic DNA hybridization-based strategies, in a combined approach.[116] For example, Allen and coworkers used a catalytic hairpin assembly (CHA)-based device was combined with paper fluidics to create an ultra-cheap and portable device.[117] Similarly, by combining LAMP with CHA-based non-enzymatic circuit, Li and coworkers increased the specificity of the LAMP reaction by introducing an "AND" gate, and detected as few as 10 molecules per microliter.[118]

3.5 Detection

Following target amplification (operational step two, as shown in Figure 3.4), the amount of target should be sufficient for actual detection to finally occur (operation step three). This step introduces many opportunities for nanotechnological innovations. Nanomaterials can function as labels for

fluorescence or electrochemical detection. Nanofunctionalized sensing surfaces can enable higher binding capacities or other unique properties. Nanoscale phenomenon such as fluorescence transfer resonance and plasmon resonance can also be utilized for detection. We address detection in three broad strategies for sensing and read-out: naked eye detection (based on lateral flow assay and NP aggregation technologies), optical detection, and electrochemical-based strategies.

3.5.1 Naked eye detection

The lateral flow assay (LFA) is the most widely used commercially available POC diagnostic format today.[9], [119] It has the key advantages of low cost, high speed, and simple format and readout – all of which make it one of the most promising POC platforms for both developing and developed worlds. A widespread example of a successful, commercially-available LFA product is the home pregnancy test device, which not only comes in a simple test-strip format but can be used by virtually anyone with little to no prior training. In addition to confirming pregnancy, LFA devices have also been developed for infectious disease detection, drug abuse screening, and rapid clinical diagnosis of serious health events such as heart attacks and strokes. Traditionally, LFA devices have

been used for immunological detection, but they have also been adapted for nucleic acid-based detection as well.[120] For example, Baumner and co-workers demonstrated nucleic acid detection in a test strip format with nanomolar detection limits,[121] and Carter and Cary demonstrated amplification in an LFA format using latex microparticles.[122] Many of the concepts and technologies of LFA have recently been extended and utilized in the context of paper-based microfluidics.[123]

In general, LFA devices are chromatographic platforms which contain functionalized particles (e.g. gold nanoparticles, latex beads, or liposomes) that bind to particular analytes in a given sample.[124] The analyte-particle complex subsequently flows laterally, driven by capillary force, through a series of overlapping membranes within the device and is finally captured in a "readout" portion of the device. A visual result (i.e. colored band) is usually achieved within a few minutes, which can be easily interpreted by an untrained individual without the need for expensive equipment. In the case of nucleic acids, LFA devices exploit the specificity of DNA hybridization to capture and detect nucleic acid targets in a manner very similar to the original LFA assays which typically detected antibodies or antigens. Nucleic acids can be captured on lateral flow test strips in several different ways. Most commonly, gold nanoparticles can be functionalized with single stranded DNA capture probes that will selectively

bind to distinct DNA targets and generate an AuNP aggregate that yields a clear color change in the test strip. For example, an innovative POC NALF device was developed by Mao and coworkers.[125] In this work a disposable nucleic acid biosensor strip with nanoparticle probes was used to perform low-cost and sensitive detection of nucleic acid targets within 15 minutes. Sandwich-type DNA hybridization reactions were employed to capture targets onto immobilized nucleic acid probes and enable gold NP binding to the captured targets. The concentration of gold NPs on the immobilized targets generates a distinct color change on the flow test strip. This detection method avoided the multiple incubation, separation, and washing steps that are often required for nucleic acid biosensors and demonstrated the successfully use of DNA-NP aggregates for nucleic acid detection within a lateral flow biosensor.

To improve the sensitivity and specificity the NALF assay format, Puchang Lie and colleagues used an isothermal strand-displacement polymerase reaction in a lateral flow strip format.[126] Similarly, Rohrman and coworkers designed a NALF device, using gold nanoparticle probes and a gold enhancement solution to monitor HIV viral load in patients over a clinically relevant range.[127]

Metal nanoparticles (particularly gold) for DNA sensing have also been utilized in a colorimetric detection scheme that is based on gold nanoparticles

aggregating together instead of binding to a surface region on a lateral flow strip. Similar to the LFA approach, this NP aggregation approach can potentially enable naked eye detection. Gold nanoparticles exhibit a color change that is dependent on the interparticle distances in solution. Mirkin and colleagues developed a colorimetric detection system utilizing gold nanoparticles containing surface-immobilized DNA probes that bound to DNA targets and yielded distinct colorimetric signals.[128] Expanding on this approach, Storhoff and co-workers spotted DNA-modified gold NPs onto a glass waveguide slide and monitored the scattering effects from the gold nanoparticles, which yields a yellow to orange color due to a plasmon band red shift.[129] Another innovative and simple method for colorimetric detection was devised by Mollasalehi et al, who used non-crosslinking gold nanoprobes that aggregated primarily via changes in ionic strength to achieve detection readouts visible to the naked eye.[130] In another approach that used ligation chain reaction as an amplification step, Shen and coworkers demonstrated gold nanoparticle aggregation for detection of DNA down to 20 attomolar concentration.[131] These examples represent how nanoparticle based colorimetric detection can meet POC criteria without sacrificing sensitivity or specificity.

3.5.2 Optical detection

Optical detection is widely employed in laboratory-based clinical chemistry, typically utilizing microscopes, lasers, spectrophotometers, lenses, and filters that are precisely arranged and aligned in the controlled laboratory environment. These systems may be difficult to translate to a portable or minimal complexity format. Fortunately, technological progress in LEDs, laser diodes, and low-cost sensors, as well as the continued development of microfluidic integration and nanoscale materials, is leading to increased feasibility and convenience for optical POC detection.

As described previously, the isothermal amplification method LAMP can be monitored by measuring turbidity, resulting from complex formation of pyrophosphate and divalent metallic ions [85] Implementing this method, Fang and colleagues used LAMP for viral nucleic acid detection within an 8-channel microfluidic chip that generated a readout that could be visualized either by naked eye or, for more quantitative measurements, via absorbance measured by an optical sensor and built-in LED.[132] As a step toward a more integrated LAMP device, Fang and researchers designed a LAMP microfluidic device which can lyse bacteria and perform LAMP reactions in parallel for multiplexed POC detection of bacteria.[133] Another interesting POC method incorporated both

fluorescence and colorimetric detection of nucleic acids, and utilized DNAzymes as the catalyst for a cleavage-based signal amplification scheme.[134] Other work has demonstrated the use of digital LAMP that could be potentially used for quantities nucleic acid detection. [135], [136]

3.5.3 Electrochemical detection

Electrochemistry-based DNA sensors offer important benefits for POC diagnostics. These sensors provide sensitivity, selectivity and low cost. A wide range of chemical methods are also available for functionalizing and immobilizing capture probes on many types of electrode substrates, enabling extra versatility in the design and chemical tuning of these DNA biosensors. In addition, electrochemical reactions provide a direct electronic signal so the sensors can bypass the need for expensive signal transduction equipment. Unlike the optical approach, electrochemical detection usually requires electrodes to be interface directly with samples, which may introduce fouling or necessitate replacement of disposable components for each test. Electrochemical detection approaches can be integrated directly with additional electronic components for signal processing or read-out, and potentially be manufactured at low cost (benefitting from the existing production capabilities of the microelectronics

industry).

DNA electrochemical sensors in general contain nucleic acid layers for capturing and detecting the nucleic acid target, and an electrochemical transducer which enables a simple, accurate readout directly from the electrochemical reactions occurring within the device. One of the important early electrochemical DNA biosensors was a nanoparticle-based electrical detection device which utilized electrochemical stripping to distinguish hybridization of a target oligonucleotide.[137] Hsieh and coworkers achieved real-time quantitative nucleic acid detection by combining microfluidics, isothermal DNA amplification methods (LAMP), and electrochemical detection into a single POC device.[138] In another work, highly sensitive detection of DNA targets was achieved using a pair of interdigitated electrodes as the impedance biosensor.[139] Fang and coworkers developed a sensitive, PCR-free, electronic chip that performed multiplex detection of a panel of mRNA to screen for prostate cancer.[34] Another great advancement in portable electrochemical POC devices was demonstrated by Toumazou *et al*., who developed a pH-sensing, CMOS-based device that performs real-time detection of nucleic acids amplified via PCR or isothermal amplification.[140] This platform was successfully used for genotyping DNA from crude human saliva samples in about 30 minutes, and it additionally was highly sensitive and can detect low copy numbers.

3.6 Conclusion and outlook

The current generation of rapid diagnostic tests, such as the immunological tests for pregnancy, strep throat, and HIV, has already demonstrated the practical benefits as well as the marketability of POC detection technology. At the same time, increased popularity of molecular diagnostics for clinical applications, as well as the increasing popularity of next generation sequencing, is driving increased interest in nucleic acid based detection methods. Nucleic acid-based tests will grow progressively widespread as new assays are introduced for diseases and conditions that have traditionally relied on other diagnostic methods. Thus, the current research and clinical climate seems promising for nucleic acid diagnostics to become increasingly prevalent and relevant for POC detection.

Despite this great potential, the field of POC detection of nucleic acids is still in its early stages. POC detection demands reliable performance and high sensitivity, the capacity to handle variable test samples with diverse physical and chemical properties, and minimal sampling handling and procedural complexity. To overcome these challenges, nanotechnology will undoubtedly play a key role in the continued development of POC assays. Progress will rely heavily on

cutting edge innovations in nanotechnology including the use of metal nanoparticles and DNA nanostructures and reaction networks, as well as nanomaterials such as carbon nanotubes, nanowires, and quantum dots. These nanoscale technologies will provide unique capabilities to enable the next generation of POC nucleic acid diagnostics.

As the portable nucleic acid detection field matures, greater emphasis must be placed on complete integration of technologies and validation of performance; the aforementioned POC ASSURED criteria may be used as guidelines for this development. Successfully meeting all or most of the ASSURED criteria will allow for important advances over a range of applications. For example, automated detection systems may be developed for crop and food safety, environmental monitoring, and bioterrorism, thus overcoming significant challenges in agriculture, food security, and public health and safety. To this end, future work will aim to achieve real-world implementation and focus on utilizing the platform technologies described here in conjunction with more realistic sample matrices and environmental conditions. By taking full advantage of nanotechnology innovations, devices for POC detection of nucleic acids will ultimately lead to substantial advancements in diagnostics and widespread use.

3.7 References

[1] L. Kulinsky, Z. Noroozi, and M. Madou, "Present technology and future trends in point-of-care microfluidic diagnostics.," *Methods Mol Biol*, vol. 949, pp. 3–23, Jan. 2013.

[2] P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam, and B. H. Weigl, "Microfluidic diagnostic technologies for global public health.," *Nature*, vol. 442, no. 7101, pp. 412–8, Jul. 2006.

[3] M. Urdea, L. A. Penny, S. S. Olmsted, M. Y. Giovanni, P. Kaspar, A. Shepherd, P. Wilson, C. A. Dahl, S. Buchsbaum, G. Moeller, and D. C. Hay Burgess, "Requirements for high impact diagnostics in the developing world," *Nature*, vol. 444, no. Suppl 1, pp. 73–9, Nov. 2006.

[4] R. W. Peeling, K. K. Holmes, D. Mabey, and A. Ronald, "Rapid tests for sexually transmitted infections (STIs): the way forward.," *Sex Transm Infect*, vol. 82 Suppl 5, pp. v1–6, Dec. 2006.

[5] C. D. Chin, V. Linder, and S. K. Sia, "Commercialization of microfluidic point-of-care diagnostic devices.," *Lab Chip*, vol. 12, no. 12, pp. 2118–34, Jun. 2012.

[6] K. Zamanian and S. Whitmore, "U.S. IVD Market to Exceed $9.5 Billion by 2017, IVD Technology," 2012.

[7] T. M.-H. Lee, "Over-the-Counter Biosensors: Past, Present, and Future," *Sensors*, vol. 8, no. 9, pp. 5535–5559, Sep. 2008.

[8] E. Lee-Lewandrowski and K. Lewandrowski, "Perspectives on cost and outcomes for point-of-care testing.," *Clin Lab Med*, vol. 29, no. 3, pp. 479–89, Sep. 2009.

[9] B. Ngom, Y. Guo, X. Wang, and D. Bi, "Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review.," *Analytical and Bioanalytical Chemistry*, vol. 397, no. 3, pp. 1113–35, Jun. 2010.

[10] V. Gubala, L. F. Harris, A. J. Ricco, M. X. Tan, and D. E. Williams, "Point of care diagnostics: status and future.," *Anal Chem*, vol. 84, no. 2, pp. 487–515, Jan. 2012.

[11] J. Huggett, C. Green, and A. Zumla, "Nucleic acid detection and quantification in the developing world.," *Biochem Soc Trans*, vol. 37, no. Pt 2, pp. 419–23, Apr. 2009.

[12] L. O'Connor and B. Glynn, "Recent advances in the development of nucleic acid diagnostics.," *Expert Rev Med Devices*, vol. 7, no. 4, pp. 529–39, Jul. 2010.

[13] J. Weile and C. Knabbe, "Current applications and future trends of molecular diagnostics in clinical bacteriology.," *Anal Bioanal Chem*, vol. 394, no. 3, pp. 731–42, Jun. 2009.

[14] K. L. Muldrew, "Molecular diagnostics of infectious diseases.," *Curr Opin Pediatr*, vol. 21, no. 1, pp. 102–11, Feb. 2009.

[15] S. L. Stramer, U. Wend, D. Candotti, G. A. Foster, F. B. Hollinger, R. Y. Dodd, J.-P. Allain, and W. Gerlich, "Nucleic acid testing to detect HBV infection in blood donors.," *The New England Journal of Medicine*, vol. 364, no. 3, pp. 236–47, Jan. 2011.

[16] E. C. Hayden, "Targeted treatment tested as potential cancer cure.," *Nature*, vol. 479, no. 7373, p. 281, Nov. 2011.

[17] L. V Sequist, R. S. Heist, A. T. Shaw, P. Fidias, R. Rosovsky, J. S. Temel, I. T. Lennes, S. Digumarthy, B. A. Waltman, E. Bast, S. Tammireddy, L. Morrissey, A. Muzikansky, S. B. Goldberg, J. Gainor, C. L. Channick, J. C. Wain, H. Gaissert, D. M. Donahue, A. Muniappan, C. Wright, H. Willers, D. J. Mathisen, N. C. Choi, J. Baselga, T. J. Lynch, L. W. Ellisen, M. Mino-Kenudson, M. Lanuti, D. R. Borger, A. J. Iafrate, J. A. Engelman, and D. Dias-Santagata, "Implementing multiplexed genotyping of non-small-cell lung cancers into routine clinical practice.," *Annals of Oncology*, vol. 22, no. 12, pp. 2616–24, Dec. 2011.

[18] World Health Organization, "Real-time RT-PCR protocol for the detection of avian influenza a (H7N9) virus," 2013.

[19] CDC, "Diagnostics for Detecting H7N9 Using rRT-PCR," 2013.

[20] J.-M. Nam, C. S. Thaxton, and C. A. Mirkin, "Nanoparticle-based bio-bar codes for the ultrasensitive detection of proteins.," *Science*, vol. 301, no. 5641, pp. 1884–6, Sep. 2003.

[21] G. Doria, J. Conde, B. Veigas, L. Giestas, C. Almeida, M. Assunção, J. Rosa, and P. V Baptista, "Noble metal nanoparticles for biosensing applications.," *Sensors*, vol. 12, no. 2, pp. 1657–87, Jan. 2012.

[22] S. J. Tan, M. J. Campolongo, D. Luo, and W. Cheng, "Building plasmonic nanostructures with DNA.," *Nature Nanotechnology*, vol. 6, no. 5, pp. 268–76, May 2011.

[23] J. B. Lee, M. J. Campolongo, J. S. Kahn, Y. H. Roh, M. R. Hartman, and D. Luo, "DNA-based nanostructures for molecular sensing.," *Nanoscale*, vol. 2, no. 2, pp. 188–97, Feb. 2010.

[24] J. B. Lee, Y. H. Roh, S. H. Um, H. Funabashi, W. Cheng, J. J. Cha, P. Kiatwuthinon, D. A. Muller, and D. Luo, "Multifunctional nanoarchitectures from DNA-based ABC monomers.," *Nature Nanotechnology*, vol. 4, no. 7, pp. 430–6, Jul. 2009.

[25] Y. Li, Y. T. H. Cu, and D. Luo, "Multiplexed detection of pathogen DNA with DNA-based fluorescence nanobarcodes.," *Nature Biotechnology*, vol. 23, no. 7, pp. 885–9, Jul. 2005.

[26] D. Y. Zhang and G. Seelig, "Dynamic DNA nanotechnology using strand-displacement reactions.," *Nat Chem*, vol. 3, no. 2, pp. 103–13, Feb. 2011.

[27] A. J. Saleh Ahammad, J.-J. Lee, and M. A. Rahman, "Electrochemical sensors based on carbon nanotubes.," *Sensors*, vol. 9, no. 4, pp. 2289–319, Jan. 2009.

[28] P. Zrazhevskiy, M. Sena, and X. Gao, "Designing multifunctional quantum dots for bioimaging, detection, and drug delivery.," *Chem Soc Rev*, vol. 39, no. 11, pp. 4326–54, Nov. 2010.

[29] U. A. Gunasekera, Q. A. Pankhurst, and M. Douek, "Imaging applications of nanotechnology in cancer.," *Target Oncol*, vol. 4, no. 3, pp. 169–81, Sep. 2009.

[30] M. Baker, "Nanotechnology imaging probes: smaller and more stable," *Nature Methods*, vol. 7, no. 12, pp. 957–962, Dec. 2010.

[31] J. Lee, P. Hernandez, J. Lee, A. O. Govorov, and N. A. Kotov, "Exciton-plasmon interactions in molecular spring assemblies of nanowires and wavelength-based protein detection.," *Nat Mater*, vol. 6, no. 4, pp. 291–5, Apr. 2007.

[32] E. D. Goluch, J.-M. Nam, D. G. Georganopoulou, T. N. Chiesl, K. A. Shaikh, K. S. Ryu, A. E. Barron, C. A. Mirkin, and C. Liu, "A bio-barcode assay for on-chip attomolar-sensitivity protein detection.," *Lab Chip*, vol. 6, no. 10, pp. 1293–9, Oct. 2006.

[33] L. Soleymani, Z. Fang, E. H. Sargent, and S. O. Kelley, "Programming the detection limits of biosensors through controlled nanostructuring.," *Nat Nanotechnol*, vol. 4, no. 12, pp. 844–8, Dec. 2009.

[34] Z. Fang, L. Soleymani, G. Pampalakis, M. Yoshimoto, J. A. Squire, E. H. Sargent, and S. O. Kelley, "Direct profiling of cancer biomarkers in tumor tissue using a multiplexed nanostructured microelectrode integrated circuit.," *ACS Nano*, vol. 3, no. 10, pp. 3207–13, Oct. 2009.

[35] J. Zhang and C. M. Li, "Nanoporous metals: fabrication strategies and advanced electrochemical applications in catalysis, sensing and energy systems.," *Chem Soc Rev*, vol. 41, no. 21, pp. 7016–31, Nov. 2012.

[36] C. J. Ingham, J. ter Maat, and W. M. de Vos, "Where bio meets nano: the many uses for nanoporous aluminum oxide in biotechnology.," *Biotechnol Adv*, vol. 30, no. 5, pp. 1089–99, 2012.

[37] S. Park, H. C. Kim, and T. D. Chung, "Electrochemical analysis based on nanoporous structures.," *Analyst*, vol. 137, no. 17, pp. 3891–903, Sep. 2012.

[38] P. Stroeve and N. Ileri, "Biotechnical and other applications of nanoporous membranes.," *Trends Biotechnol*, vol. 29, no. 6, pp. 259–66, Jun. 2011.

[39] D. Yang, X. Niu, Y. Liu, Y. Wang, X. Gu, L. Song, R. Zhao, L. Ma, Y. Shao, and X. Jiang, "Electrospun Nanofibrous Membranes: A Novel Solid Substrate for Microfluidic Immunoassays for HIV," *Advanced Materials*, vol. 20, no. 24, pp. 4770–4775, Dec. 2008.

[40] D. A. Giljohann and C. A. Mirkin, "Drivers of biodiagnostic development.," *Nature*, vol. 462, no. 7272, pp. 461–4, Nov. 2009.

[41] S. Wang, F. Inci, G. De Libero, A. Singhal, and U. Demirci, "Point-of-care assays for tuberculosis: role of nanotechnology/microfluidics.," *Biotechnol Adv*, vol. 31, no. 4, pp. 438–49, Jul. 2013.

[42] X. Zhang, Q. Guo, and D. Cui, "Recent advances in nanotechnology applied to biosensors.," *Sensors*, vol. 9, no. 2, pp. 1033–53, Jan. 2009.

[43] K. M. Abu-Salah, S. A. Alrokyan, M. N. Khan, and A. A. Ansari, "Nanomaterials as analytical tools for genosensors.," *Sensors*, vol. 10, no. 1, pp. 963–93, Jan. 2010.

[44] M. A. Dineva, L. MahiLum-Tapay, and H. Lee, "Sample preparation: a challenge in the development of point-of-care nucleic acid-based assays for resource-limited settings.," *Analyst*, vol. 132, no. 12, pp. 1193–9, Dec. 2007.

[45] M. Ritzi-Lehnert, "Development of chip-compatible sample preparation for diagnosis of infectious diseases.," *Expert Rev Mol Diagn*, vol. 12, no. 2, pp. 189–206, Mar. 2012.

[46] R. Mariella, "Sample preparation: the weak link in microfluidics-based biodetection.," *Biomed Microdevices*, vol. 10, no. 6, pp. 777–84, Dec. 2008.

[47] J. Wen, L. A. Legendre, J. M. Bienvenue, and J. P. Landers, "Purification of nucleic acids in microfluidic devices.," *Anal Chem*, vol. 80, no. 17, pp. 6472–9, Sep. 2008.

[48] A. Puren, J. L. Gerlach, B. H. Weigl, D. M. Kelso, and G. J. Domingo, "Laboratory operations, specimen processing, and handling for viral load testing and surveillance.," *J Infect Dis*, vol. 201, no. Suppl, pp. S27–36, Apr. 2010.

[49] M. M. Rahman and A. Elaissari, "Nucleic acid sample preparation for in vitro molecular diagnosis: from conventional techniques to biotechnology.," *Drug Discov Today*, vol. 17, no. 21–22, pp. 1199–207, Nov. 2012.

[50] Z. Lin and Z. Cai, "Cell lysis methods for high-throughput screening or miniaturized assays.," *Biotechnol J*, vol. 4, no. 2, pp. 210–5, Feb. 2009.

[51] K. H. Cheong, D. K. Yi, J.-G. Lee, J.-M. Park, M. J. Kim, J. B. Edel, and C. Ko, "Gold nanoparticles for one step DNA extraction and real-time PCR of pathogens in a single chamber.," *Lab Chip*, vol. 8, no. 5, pp. 810–3, May 2008.

[52] H. Schwarzenbach, D. S. B. Hoon, and K. Pantel, "Cell-free nucleic acids as biomarkers in cancer patients.," *Nat Rev Cancer*, vol. 11, no. 6, pp. 426–37, Jun. 2011.

[53] V. Swarup and M. R. Rajeswari, "Circulating (cell-free) nucleic acids--a promising, non-invasive tool for early detection of several human diseases.," *FEBS Lett*, vol. 581, no. 5, pp. 795–9, Mar. 2007.

[54] E. M. Shekhtman, K. Anne, H. S. Melkonyan, D. J. Robbins, S. L. Warsof, and S. R. Umansky, "Optimization of transrenal DNA analysis: detection of fetal DNA in maternal urine.," *Clin Chem*, vol. 55, no. 4, pp. 723–9, Apr. 2009.

[55] C. Schrader, A. Schielke, L. Ellerbroek, and R. Johne, "PCR inhibitors - occurrence, properties and removal.," *J Appl Microbiol*, vol. 113, no. 5, pp. 1014–26, Nov. 2012.

[56] R. Boom, C. J. Sol, M. M. Salimans, C. L. Jansen, P. M. Wertheim-van Dillen, and J. van der Noordaa, "Rapid and simple method for purification of nucleic acids.," *J. Clin. Microbiol.*, vol. 28, no. 3, pp. 495–503, Mar. 1990.

[57] L. Zhang, S. M. Rafei, L. Xie, M. B.-R. Chew, H. M. Ji, Y. Chen, R. Rajoo, K.-L. Ong, R. Tan, S.-H. Lau, V. T. K. Chow, C.-K. Heng, K.-H. Teo, and T. G. Kang, "A self-contained disposable cartridge microsystem for dengue viral ribonucleic acid extraction," *Sensors and Actuators B: Chemical*, vol. 160, no. 1, pp. 1557–1564, Dec. 2011.

[58] S. Huang, J. Do, M. Mahalanabis, A. Fan, L. Zhao, L. Jepeal, S. K. Singh, and C. M. Klapperich, "Low cost extraction and isothermal amplification of DNA for infectious diarrhea diagnosis.," *PLoS One*, vol. 8, no. 3, p. e60059, Jan. 2013.

[59] M. Mahalanabis, J. Do, H. ALMuayad, J. Y. Zhang, and C. M. Klapperich, "An integrated disposable device for DNA extraction and helicase dependent amplification.," *Biomed Microdevices*, vol. 12, no. 2, pp. 353–9, Apr. 2010.

[60] S. R. Jangam, D. H. Yamada, S. M. McFall, and D. M. Kelso, "Rapid, point-of-care extraction of human immunodeficiency virus type 1 proviral DNA from whole blood for detection by real-time PCR.," *J Clin Microbiol*, vol. 47, no. 8, pp. 2363–8, Aug. 2009.

[61] A. V Govindarajan, S. Ramachandran, G. D. Vigil, P. Yager, and K. F. Böhringer, "A low cost point-of-care viscous sample preparation device for molecular diagnosis in the developing world; an example of microfluidic origami.," *Lab Chip*, vol. 12, no. 1, pp. 174–81, Jan. 2012.

[62] D. Lehmann, S. Jouette, F. Olivieri, S. Laborde, C. Rofel, E. Simon, D. Metz, L. Felden, and S. Ribault, "Novel sample preparation method for molecular detection of Mollicutes in cell culture samples.," *J Microbiol Methods*, vol. 80, no. 2, pp. 183–9, Feb. 2010.

[63] H. Zhang, H. H. Tran, B. H. Chung, and N. Y. Lee, "Solid-phase based on-chip DNA purification through a valve-free stepwise injection of multiple reagents employing centrifugal force combined with a hydrophobic capillary barrier pressure.," *Analyst*, vol. 138, no. 6, pp. 1750–7, Mar. 2013.

[64] C. R. Reedy, C. W. Price, J. Sniegowski, J. P. Ferrance, M. Begley, and J. P. Landers, "Solid phase extraction of DNA from biological samples in a post-based, high surface area poly(methyl methacrylate) (PMMA) microdevice.," *Lab Chip*, vol. 11, no. 9, pp. 1603–11, May 2011.

[65] E. A. Oblath, W. H. Henley, J. P. Alarie, and J. M. Ramsey, "A microfluidic chip integrating DNA extraction and real-time PCR for the detection of bacteria in saliva.," *Lab Chip*, vol. 13, no. 7, pp. 1325–32, Apr. 2013.

[66] G. Hennig, C. Petry, and E. Sampson, "Automating Nucleic Acid Isolation for In Vitro Use Provides Improved Assay Performance in the Molecular Diagnostics Lab," 2010.

[67] G. R. M. Duarte, C. W. Price, J. L. Littlewood, D. M. Haverstick, J. P. Ferrance, E. Carrilho, and J. P. Landers, "Characterization of dynamic solid phase DNA extraction from blood with magnetically controlled silica beads.," *Analyst*, vol. 135, no. 3, pp. 531–7, Mar. 2010.

[68] K. Sur, S. M. McFall, E. T. Yeh, S. R. Jangam, M. A. Hayden, S. D. Stroupe, and D. M. Kelso, "Immiscible phase nucleic acid purification eliminates

PCR inhibitors with a single pass of paramagnetic particles through a hydrophobic liquid.," *J Mol Diagn*, vol. 12, no. 5, pp. 620–8, Sep. 2010.

[69] S. M. Berry, E. T. Alarid, and D. J. Beebe, "One-step purification of nucleic acid for gene expression analysis via Immiscible Filtration Assisted by Surface Tension (IFAST).," *Lab Chip*, vol. 11, no. 10, pp. 1747–53, May 2011.

[70] H. Bordelon, N. M. Adams, A. S. Klemm, P. K. Russ, J. V Williams, H. K. Talbot, D. W. Wright, and F. R. Haselton, "Development of a low-resource RNA extraction cassette based on surface tension valves.," *ACS Applied Materials & Interfaces*, vol. 3, no. 6, pp. 2161–8, Jun. 2011.

[71] R. C. den Dulk, K. A. Schmidt, G. Sabatté, S. Liébana, and M. W. J. Prins, "Magneto-capillary valve for integrated purification and enrichment of nucleic acids and proteins.," *Lab Chip*, vol. 13, no. 1, pp. 106–18, Jan. 2013.

[72] P. Craw and W. Balachandran, "Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review.," *Lab Chip*, vol. 12, no. 14, pp. 2469–86, Jul. 2012.

[73] C.-C. Chang, C.-C. Chen, S.-C. Wei, H.-H. Lu, Y.-H. Liang, and C.-W. Lin, "Diagnostic devices for isothermal nucleic acid amplification.," *Sensors*, vol. 12, no. 6, pp. 8319–37, Jan. 2012.

[74] P. J. Asiello and A. J. Baeumner, "Miniaturized isothermal nucleic acid amplification, a review.," *Lab Chip*, vol. 11, no. 8, pp. 1420–30, Apr. 2011.

[75] S. Park, Y. Zhang, S. Lin, T.-H. Wang, and S. Yang, "Advances in microfluidic PCR for point-of-care infectious disease diagnostics.," *Biotechnol Adv*, vol. 29, no. 6, pp. 830–9, 2011.

[76] C. Zhang and D. Xing, "Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends.," *Nucleic Acids Res*, vol. 35, no. 13, pp. 4223–37, Jan. 2007.

[77] A. Van Rie, L. Page-Shipp, L. Scott, I. Sanne, and W. Stevens, "Xpert(®) MTB/RIF for point-of-care diagnosis of TB in high-HIV burden, resource-limited countries: hype or hope?," *Expert Rev Mol Diagn*, vol. 10, no. 7, pp. 937–46, Oct. 2010.

[78] G. Maltezos, M. Johnston, K. Taganov, C. Srichantaratsamee, J. Gorman, D. Baltimore, W. Chantratita, and A. Scherer, "Exploring the limits of ultrafast polymerase chain reaction using liquid for thermal heat exchange: A proof of principle.," *Appl Phys Lett*, vol. 97, no. 26, p. 264101, Dec. 2010.

[79] S. R. Jangam, A. K. Agarwal, K. Sur, and D. M. Kelso, "A point-of-care PCR test for HIV-1 detection in resource-limited settings.," *Biosens Bioelectron*, vol. 42, no. null, pp. 69–75, Apr. 2013.

[80] T. Notomi, "Loop-mediated isothermal amplification of DNA," *Nucleic Acids Research*, vol. 28, no. 12, p. 63e–63, Jun. 2000.

[81] R. Abdul-Ghani, A. M. Al-Mekhlafi, and P. Karanis, "Loop-mediated isothermal amplification (LAMP) for malarial parasites of humans: would it come to clinical reality as a point-of-care test?," *Acta Trop*, vol. 122, no. 3, pp. 233–40, Jun. 2012.

[82] X. Zhang, M. Liao, P. Jiao, K. Luo, H. Zhang, T. Ren, G. Zhang, C. Xu, C. Xin, and W. Cao, "Development of a loop-mediated isothermal amplification assay for rapid detection of subgroup J avian leukosis virus.," *J Clin Microbiol*, vol. 48, no. 6, pp. 2116–21, Jun. 2010.

[83] B. Sun, F. Shen, S. E. McCalla, J. E. Kreutz, M. A. Karymov, and R. F. Ismagilov, "Mechanistic evaluation of the pros and cons of digital RT-LAMP for HIV-1 viral load quantification on a microfluidic device and improved efficiency via a two-step digital protocol.," *Anal Chem*, vol. 85, no. 3, pp. 1540–6, Feb. 2013.

[84] N. W. Lucchi, A. Demas, J. Narayanan, D. Sumari, A. Kabanywanyi, S. P. Kachur, J. W. Barnwell, and V. Udhayakumar, "Real-time fluorescence loop mediated isothermal amplification for the diagnosis of malaria.," *PLoS One*, vol. 5, no. 10, p. e13733, Jan. 2010.

[85] N. Tomita, Y. Mori, H. Kanda, and T. Notomi, "Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products.," *Nat Protoc*, vol. 3, no. 5, pp. 877–82, Jan. 2008.

[86] Z. K. Njiru, "Loop-mediated isothermal amplification technology: towards point of care diagnostics.," *PLoS Negl Trop Dis*, vol. 6, no. 6, p. e1572, Jan. 2012.

[87] J. Compton, "Nucleic acid sequence-based amplification.," *Nature*, vol. 350, no. 6313, pp. 91–2, Mar. 1991.

[88] X. Zhao and T. Dong, "Multifunctional sample preparation kit and on-chip quantitative nucleic acid sequence-based amplification tests for microbial detection.," *Anal Chem*, vol. 84, no. 20, pp. 8541–8, Oct. 2012.

[89] A. Mader, U. Riehle, T. Brandstetter, E. Stickeler, and J. Ruehe, "Universal nucleic acid sequence-based amplification for simultaneous amplification of messengerRNAs and microRNAs.," *Anal Chim Acta*, vol. 754, pp. 1–7, Nov. 2012.

[90] P. M. Lizardi, X. Huang, Z. Zhu, P. Bray-Ward, D. C. Thomas, and D. C. Ward, "Mutation detection and single-molecule counting using isothermal rolling-circle amplification.," *Nat Genet*, vol. 19, no. 3, pp. 225–32, Jul. 1998.

[91] D. Liu, S. L. Daubendiek, M. a Zillman, K. Ryan, and E. T. Kool, "Rolling Circle DNA Synthesis: Small Circular Oligonucleotides as Efficient Templates for DNA Polymerases.," *J Am Chem Soc*, vol. 118, no. 7, pp. 1587–1594, Feb. 1996.

[92] Y. Zhou, Q. Huang, J. Gao, J. Lu, X. Shen, and C. Fan, "A dumbbell probe-mediated rolling circle amplification strategy for highly sensitive microRNA detection.," *Nucleic Acids Res*, vol. 38, no. 15, p. e156, Aug. 2010.

[93] D. Y. Zhang, W. Zhang, X. Li, and Y. Konomi, "Detection of rare DNA targets by isothermal ramification amplification.," *Gene*, vol. 274, no. 1–2, pp. 209–16, Aug. 2001.

[94] S. Bi, Y. Cui, and L. Li, "Dumbbell probe-mediated cascade isothermal amplification: a novel strategy for label-free detection of microRNAs and its application to real sample assay.," *Anal Chim Acta*, vol. 760, pp. 69–74, Jan. 2013.

[95] M. Vincent, Y. Xu, and H. Kong, "Helicase-dependent isothermal DNA amplification.," *EMBO Rep*, vol. 5, no. 8, pp. 795–800, Aug. 2004.

[96] A. Motré, R. Kong, and Y. Li, "Improving isothermal DNA amplification speed for the rapid detection of Mycobacterium tuberculosis.," *J Microbiol Methods*, vol. 84, no. 2, pp. 343–5, Feb. 2011.

[97] P. Gill, A.-H. Alvandi, H. Abdul-Tehrani, and M. Sadeghizadeh, "Colorimetric detection of Helicobacter pylori DNA using isothermal helicase-dependent amplification and gold nanoparticle probes.," *Diagn Microbiol Infect Dis*, vol. 62, no. 2, pp. 119–24, Oct. 2008.

[98] J. Goldmeyer, H. Kong, and W. Tang, "Development of a novel one-tube isothermal reverse transcription thermophilic helicase-dependent amplification platform for rapid RNA detection.," *J Mol Diagn*, vol. 9, no. 5, pp. 639–44, Nov. 2007.

[99] L. An, W. Tang, T. a Ranalli, H.-J. Kim, J. Wytiaz, and H. Kong, "Characterization of a thermostable UvrD helicase and its participation in helicase-dependent amplification.," *J Biol Chem*, vol. 280, no. 32, pp. 28952–8, Aug. 2005.

[100] F. Kivlehan, F. Mavré, L. Talini, B. Limoges, and D. Marchal, "Real-time electrochemical monitoring of isothermal helicase-dependent amplification of nucleic acids.," *Analyst*, vol. 136, no. 18, pp. 3635–42, Sep. 2011.

[101] W. P. Hofmann, V. Dries, E. Herrmann, B. Gärtner, S. Zeuzem, and C. Sarrazin, "Comparison of transcription mediated amplification (TMA) and reverse transcription polymerase chain reaction (RT-PCR) for detection of hepatitis C virus RNA in liver tissue.," *J Clin Virol*, vol. 32, no. 4, pp. 289–93, Apr. 2005.

[102] G. T. Walker, M. S. Fraiser, J. L. Schram, M. C. Little, J. G. Nadeau, and D. P. Malinowski, "Strand displacement amplification--an isothermal, in vitro DNA amplification technique.," *Nucleic Acids Res*, vol. 20, no. 7, pp. 1691–6, Apr. 1992.

[103] C. Spits, C. Le Caignec, M. De Rycke, L. Van Haute, A. Van Steirteghem, I. Liebaers, and K. Sermon, "Whole-genome multiple displacement amplification from single cells.," *Nat Protoc*, vol. 1, no. 4, pp. 1965–70, Jan. 2006.

[104] O. Piepenburg, C. H. Williams, D. L. Stemple, and N. A. Armes, "DNA detection using recombination proteins.," *PLoS Biol*, vol. 4, no. 7, p. e204, Jul. 2006.

[105] G. Xu, L. Hu, H. Zhong, H. Wang, S.-I. Yusa, T. C. Weiss, P. J. Romaniuk, S. Pickerill, and Q. You, "Cross priming amplification: mechanism and

optimization for isothermal DNA amplification.," *Scientific reports*, vol. 2, p. 246, Jan. 2012.

[106] A. Niemz, T. M. Ferguson, and D. S. Boyle, "Point-of-care nucleic acid testing for infectious diseases.," *Trends Biotechnol*, vol. 29, no. 5, pp. 240–50, May 2011.

[107] B. Yurke, A. J. Turberfield, A. P. Mills, F. C. Simmel, and J. L. Neumann, "A DNA-fuelled molecular machine made of DNA.," *Nature*, vol. 406, no. 6796, pp. 605–8, Aug. 2000.

[108] G. Seelig, B. Yurke, and E. Winfree, "Catalyzed relaxation of a metastable DNA fuel.," *J Am Chem Soc*, vol. 128, no. 37, pp. 12211–20, Sep. 2006.

[109] G. Seelig, D. Soloveichik, D. Y. Zhang, and E. Winfree, "Enzyme-free nucleic acid logic circuits.," *Science*, vol. 314, no. 5805, pp. 1585–8, Dec. 2006.

[110] L. Qian and E. Winfree, "Scaling up digital circuit computation with DNA strand displacement cascades.," *Science*, vol. 332, no. 6034, pp. 1196–201, Jun. 2011.

[111] D. Y. Zhang, A. J. Turberfield, B. Yurke, and E. Winfree, "Engineering entropy-driven reactions and networks catalyzed by DNA.," *Science*, vol. 318, no. 5853, pp. 1121–5, Nov. 2007.

[112] R. M. Dirks and N. A. Pierce, "Triggered amplification by hybridization chain reaction.," *Proc Natl Acad Sci U S A*, vol. 101, no. 43, pp. 15275–8, Oct. 2004.

[113] H. M. T. Choi, J. Y. Chang, L. A. Trinh, J. E. Padilla, S. E. Fraser, and N. A. Pierce, "Programmable in situ amplification for multiplexed imaging of mRNA expression.," *Nature Biotechnology*, vol. 28, no. 11, pp. 1208–12, Nov. 2010.

[114] P. Yin, H. M. T. Choi, C. R. Calvert, and N. A. Pierce, "Programming biomolecular self-assembly pathways.," *Nature*, vol. 451, no. 7176, pp. 318–22, Jan. 2008.

[115] B. Li, A. D. Ellington, and X. Chen, "Rational, modular adaptation of enzyme-free DNA circuits to multiple detection methods.," *Nucleic Acids Res*, vol. 39, no. 16, p. e110, Sep. 2011.

[116] Y. S. Jiang, B. Li, J. N. Milligan, S. Bhadra, and A. D. Ellington, "Real-time detection of isothermal amplification reactions with thermostable catalytic hairpin assembly.," *J Am Chem Soc*, vol. 135, no. 20, pp. 7430–3, May 2013.

[117] P. B. Allen, S. A. Arshad, B. Li, X. Chen, and A. D. Ellington, "DNA circuits as amplifiers for the detection of nucleic acids on a paperfluidic platform.," *Lab Chip*, vol. 12, no. 16, pp. 2951–8, Aug. 2012.

[118] B. Li, X. Chen, and A. D. Ellington, "Adapting enzyme-free DNA circuits to the detection of loop-mediated isothermal amplification reactions.," *Analytical Chemistry*, vol. 84, no. 19, pp. 8371–7, Oct. 2012.

[119] R. J. Davies, S. S. Eapen, and S. J. Carlisle, "Lateral-Flow Immunochromatographic Assays," in *Handbook of Biosensors and Biochips*, R. S. Marks, D. C. Cullen, I. Karube, C. R. Lowe, and H. H. Weetall, Eds. Hoboken, N.J.: John Wiley, 2007, pp. 1151–1166.

[120] K. Glynou, P. C. Ioannou, T. K. Christopoulos, and V. Syriopoulou, "Oligonucleotide-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for DNA analysis by hybridization.," *Anal Chem*, vol. 75, no. 16, pp. 4155–60, Aug. 2003.

[121] A. J. Baeumner, J. Pretz, and S. Fang, "A universal nucleic acid sequence biosensor with nanomolar detection limits.," *Anal Chem*, vol. 76, no. 4, pp. 888–94, Feb. 2004.

[122] D. J. Carter and R. B. Cary, "Lateral flow microarrays: a novel platform for rapid nucleic acid detection based on miniaturized lateral flow chromatography.," *Nucleic Acids Res*, vol. 35, no. 10, p. e74, Jan. 2007.

[123] A. K. Yetisen, M. S. Akram, and C. R. Lowe, "Paper-based microfluidic point-of-care diagnostic devices.," *Lab Chip*, vol. 13, no. 12, pp. 2210–51, Jun. 2013.

[124] L. M. Zanoli, R. D'Agata, and G. Spoto, "Functionalized gold nanoparticles for ultrasensitive DNA detection.," *Anal Bioanal Chem*, vol. 402, no. 5, pp. 1759–71, Feb. 2012.

[125] X. Mao, Y. Ma, A. Zhang, L. Zhang, L. Zeng, and G. Liu, "Disposable nucleic acid biosensors based on gold nanoparticle probes and lateral flow strip.," *Anal Chem*, vol. 81, no. 4, pp. 1660–8, Feb. 2009.

[126] P. Lie, J. Liu, Z. Fang, B. Dun, and L. Zeng, "A lateral flow biosensor for detection of nucleic acids with high sensitivity and selectivity.," *Chem Commun (Camb)*, vol. 48, no. 2, pp. 236–8, Jan. 2012.

[127] B. A. Rohrman, V. Leautaud, E. Molyneux, and R. R. Richards-Kortum, "A lateral flow assay for quantitative detection of amplified HIV-1 RNA.," *PLoS One*, vol. 7, no. 9, p. e45611, Jan. 2012.

[128] C. A. Mirkin, R. L. Letsinger, R. C. Mucic, and J. J. Storhoff, "A DNA-based method for rationally assembling nanoparticles into macroscopic materials.," *Nature*, vol. 382, no. 6592, pp. 607–9, Aug. 1996.

[129] J. J. Storhoff, A. D. Lucas, V. Garimella, Y. P. Bao, and U. R. Müller, "Homogeneous detection of unamplified genomic DNA sequences based on colorimetric scatter of gold nanoparticle probes.," *Nat Biotechnol*, vol. 22, no. 7, pp. 883–7, Jul. 2004.

[130] H. Mollasalehi and R. Yazdanparast, "Non-crosslinking gold nanoprobes for detection of nucleic acid sequence-based amplification products.," *Anal Biochem*, vol. 425, no. 2, pp. 91–5, Jun. 2012.

[131] W. Shen, H. Deng, and Z. Gao, "Gold nanoparticle-enabled real-time ligation chain reaction for ultrasensitive detection of DNA.," *J Am Chem Soc*, vol. 134, no. 36, pp. 14678–81, Sep. 2012.

[132] X. Fang, Y. Liu, J. Kong, and X. Jiang, "Loop-mediated isothermal amplification integrated on microfluidic chips for point-of-care quantitative detection of pathogens.," *Anal Chem*, vol. 82, no. 7, pp. 3002–6, Apr. 2010.

[133] X. Fang, H. Chen, L. Xu, X. Jiang, W. Wu, and J. Kong, "A portable and integrated nucleic acid amplification microfluidic chip for identifying bacteria.," *Lab Chip*, vol. 12, no. 8, pp. 1495–9, Apr. 2012.

[134] Y. Zhao, L. Zhou, and Z. Tang, "Cleavage-based signal amplification of RNA.," *Nat Commun*, vol. 4, p. 1493, Jan. 2013.

[135] Q. Zhu, Y. Gao, B. Yu, H. Ren, L. Qiu, S. Han, W. Jin, Q. Jin, and Y. Mu, "Self-priming compartmentalization digital LAMP for point-of-care.," *Lab Chip*, vol. 12, no. 22, pp. 4755–63, Nov. 2012.

[136] A. Gansen, A. M. Herrick, I. K. Dimov, L. P. Lee, and D. T. Chiu, "Digital LAMP in a sample self-digitization (SD) chip.," *Lab Chip*, vol. 12, no. 12, pp. 2247–54, Jun. 2012.

[137] J. Wang, D. Xu, A. N. Kawde, and R. Polsky, "Metal nanoparticle-based electrochemical stripping potentiometric detection of DNA hybridization.," *Anal Chem*, vol. 73, no. 22, pp. 5576–81, Nov. 2001.

[138] K. Hsieh, A. S. Patterson, B. S. Ferguson, K. W. Plaxco, and H. T. Soh, "Rapid, sensitive, and quantitative detection of pathogenic DNA at the point of care through microfluidic electrochemical quantitative loop-mediated isothermal amplification.," *Angewandte Chemie International Edition*, vol. 51, no. 20, pp. 4896–900, May 2012.

[139] X. Fang, Q. Jin, F. Jing, H. Zhang, F. Zhang, H. Mao, B. Xu, and J. Zhao, "Integrated biochip for label-free and real-time detection of DNA amplification by contactless impedance measurements based on interdigitated electrodes.," *Biosens Bioelectron*, vol. 44, pp. 241–7, Jun. 2013.

[140] C. Toumazou, L. M. Shepherd, S. C. Reed, G. I. Chen, A. Patel, D. M. Garner, C.-J. A. Wang, C.-P. Ou, K. Amin-Desai, P. Athanasiou, H. Bai, I. M. Q. Brizido, B. Caldwell, D. Coomber-Alford, P. Georgiou, K. S. Jordan, J. C. Joyce, M. La Mura, D. Morley, S. Sathyavruthan, S. Temelso, R. E. Thomas, and L. Zhang, "Simultaneous DNA amplification and detection using a pH-sensing semiconductor system," *Nature Methods*, Jun. 2013.

CHAPTER 4

HYBRIDIZATION-DRIVEN AGGREGATION

4.1 Priorities and challenges of point of care diagnostics

Point of care (POC) detection is defined as diagnosis at or near the site of patient care. POC diagnostic tests could potentially offer reduced healthcare costs and increased access to testing, with extremely important benefits particularly for developing world settings.[1], [2] The development of diagnostic tests for resource limited settings requires a different set of characteristics and priorities from conventional detection methods that were originally developed to be carried out in more resource-rich environments. The acronym ASSURED, as shown in Table 4.1, is used to illustrate the attributes of a successful POC diagnostic test. A good example of a current detection methods that may be considered to meet the ASSURED criteria are immunological-based rapid tests such as the well-established instant pregnancy test based on detection of Human Chorionic Gonadotropin (hCG), which serves as a ubiquitous and popular demonstration of the marketability and benefits associated with POC testing.

Affordable	Increase access to testing by lowering cost barrier; especially important for developing world setting
Sensitive	Detect targets down to low concentrations for enhanced diagnostic performance
Specific	Minimize false positives by selective recognition of the target
User-friendly	Low complexity assay to be carried out by minimally-trained personnel
Rapid and **R**obust	Deliver rapid time-to-answer, assay should be reliable performance even in non-ideal conditions
Equipment-free or minimal equipment	Emphasize portability and ease-of-use by avoiding bulky or burdensome equipment
Delivered to the greatest need	Facilitate by prioritizing easy transportation and storage of all components of the assay

Table 4.1: The ASSURED criteria describing the idealized attributes of a successful POC diagnostic test. These characteristics are useful for establishing priorities for development of new POC diagnostic assays.

However, many current diagnostic technologies require elaborate laboratory infrastructure, trained personnel, costly reagents, and components that require

special handling and storage.[3], [4] For example, nucleic-acid amplification tests such as polymerase chain reaction (PCR) can identify pathogens rapidly and with high sensitivity. In addition, nucleic acid tests are highly specific due to their ability to detect pathogens based on their unique genetic makeup.

However, most nucleic-acid amplification tests rely upon enzymatic amplification. These are not ideal for POC testing because enzymes are difficult and expensive to produce and often exhibit diminished activity under harsh conditions. In addition, carrying out these assays typically requires trained personnel and special equipment such as thermal cyclers.

Various enzyme-free methods have been demonstrated using principles derived from DNA computing and self-assembly.[5–9] Another enzyme-free approach employed aggregation of gold nanoparticles as a sensitive amplification approach for nucleic acid detection.[10] Aggregation and directed assembly of DNA-coated microbeads has been demonstrated[11], [12] and various works have since been published on the assembly of nanoparticles using DNA as a linker.[13]

4.2 Polyvalent binding of reactants enables agglutination

Agglutination is a fundamental biological process at both the micrometer and nanometer scale. At the micrometer scale, for example, red blood cells clump together in the presence of specific antibodies (hemagglutination, as occurs in a mixture of mis-matched blood types). This naturally-occurring process of agglutination has been utilized for the development of immunological bead-based assays, which are widely used for testing blood samples. Agglutination typically results in the formation of large precipitates that settle out of the solution, and this behavior can frequently be observed by the naked eye. Thus, agglutination is a convenient approach for diagnostics, because the clumping reaction occurs rapidly and is straightforward both to produce and to interpret. At the nanometer scale, agglutination plays an important role in immunological processes. Antibodies frequently have multiple sites for antigen binding which may result in aggregation and an amplified immune response. This process functions effectively as a type of signal amplification in which the local concentrations of binding reactants are dramatically increased.

The above examples of agglutination are facilitated by the polyvalent nature of the reactants. Polyvalency is an exquisite way to increase the effective strength of interactions among reactants, enabling aggregation-based assembly.[14] In

particular, polyclonal antibody-antigen binding relies on multiple binding sites of antibody interacting with antigen. However, polyvalency has not been explicitly utilized in nucleic acid-based assays, due to the fact that most DNA is linear or circular and hence is typically treated as monovalent probes or primers in conventional nucleic acid detection methods.

In this Chapter, the concept of agglutination is extended from antibody-antigen interactions to nucleic acid hybridization, and the use of DNA hybridization for aggregation phenomenon is explored with possible applications in molecular diagnostics. DNA-based agglutination using microbeads has been previously demonstrated.[11], [12] Similarly, aggregation of DNA-capped gold nanoparticles was pioneered by Mirkin as a novel and ultrasensitive method for nucleic acid detection.[10] Various works have been published on the underlying theory and characterization of gold nanoparticles assembly using DNA as a linker.[13]

In contrast to these works, our hybridization-driven aggregates are formed entirely from DNA without any additional materials (no beads or nanoparticles). Importantly, this approach can be easily interfaced with multiple detection modalities, including gel electrophoresis, optical (both bright field and fluorescence) microscopy, atomic force microscopy, dynamic light scattering

(DLS), fluorescent correlation spectroscopy (FCS), and electrochemical detection. Thus, this approach suggests a flexible platform technology that can be easily modified and further integrated in multiple formats depending on the desired real-world applications.

The free energy of a DNA duplex can be estimated, according to its sequence, using the nearest neighbor model.[15] However, hybridization-driven aggregation is theoretically more complex and polyvalent binding behavior becomes difficult to model. Thus, in this work, we relied heavily on empirical testing to determine the characteristics of hybridization-driven aggregation.

4.3 Hybridization-driven aggregation

To overcome the limitations of current nucleic acid detection methodologies and work toward POC diagnostics, we have developed a nucleic acid-based detection platform that enables signal amplification via hybridization instead of via an enzymatic reaction. This concept was based on the target-driven detection method using ABC monomers, previously developed in our laboratory.[16] In contrast to existing branched DNA (bDNA) assays,[17], [18] our hybridization-driven aggregation approach does not use surface-based hybridization or

enzyme labeling, both of which introduce additional procedural complexity, which is extremely undesirable for point-of-care applications.

Our approach, as shown in Figure 4.1, involves the use of cross-linked amplification (CLAMP) structures for target-driven hybridization and aggregation. Each CLAMP structure is designed to contain a simple Y-shaped branched DNA core with single-stranded capture probes for recognition of a specific pathogen biomarker (nucleic acid). The recognition step simply involves mixing our CLAMP structures with a patient sample containing the target biomarker. This capture probe can then bind to specific nucleic acids (ssDNA or mRNA) through the design of complementary sequences for base-pairing. After mixing, the DNA monomers and target biomarkers in the patient sample rapidly recognize and bind within several minutes due to the fast kinetics of solution-phase hybridization, and the CLAMP structures become linked together, self-assembling in the presence of a specific pathogen DNA target. However, if the target pathogen DNA is absent, there is nothing to link the DNA monomers together, and aggregation does not occur.

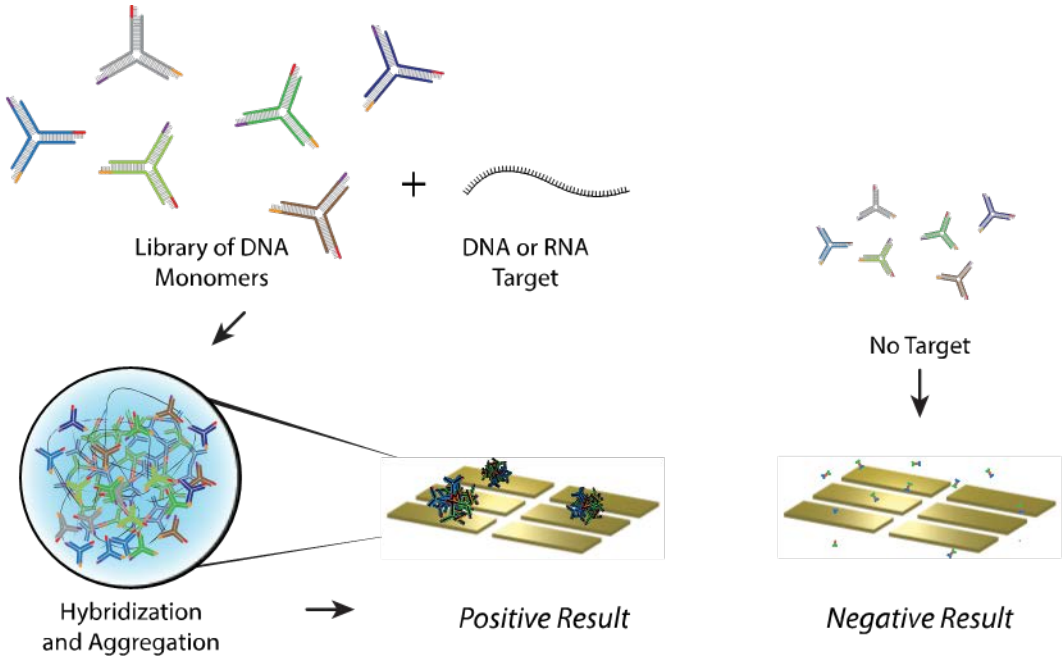


Figure 4.1: Scheme illustrating the hybridization-based DNA aggregation approach. In the presence of target, the branched DNA probes bind to multiple target copies while each target binds to multiple branched DNA probes. The resulting hybridization leads to aggregation. In the absence of target, no hybridization occurs and no aggregation takes place. A novel electrochemical sensor is used to distinguish between the relatively large aggregates and much smaller branched DNA structures which remain dissociated in solution.

The effect of aggregation is an increase in local concentration, which can be considered as an amplification of signal. After the amplification step, we carry out a detection step to distinguish between the large aggregates and the smaller DNA monomers. In this stage of our assay, we can carry out detection via several

different methods, as previously mentioned, including gel electrophoresis, dynamic light scattering, electrochemical detection, and microscopy.

4.4 Target selection and sequence design

As a prototypical target pathogen, we selected human immunodeficiency virus (HIV) due to its importance in the developing world. We selected a target region within a conserved region of the HIV genome based on the NCBI database and literature.[19] The sequence we selected is also used within a hybridization probe for an FDA-approved RT-PCR assay. We designed complementary DNA capture probes to be partially complementary to this target region and we appended these capture probe sequences to our Y-shaped DNA structural sequences. Sequences used in this experiment are shown in Table 4.2.

HIV target	TGTTAAAA**GAGACCATCAATGAGGA** **A*GCTGCAGAATGGGAT***AGATTGCAT	50 bases
Probe 3′	ATCCCATTCTGCAGC	15 bases, Tm 48.7oC
Probe 5′	TTCCTCATTGATGGTCTCTTTT	18 bases, Tm 48.5oC

Table 4.2: Set of HIV target and probe sequences. Binds to a region of the HIV-1 gag gene sequence, which is highly conserved[19]. According to BLAST, the target sequence used here is a perfect match to nearly 1000 known HIV-1 sequences (e.g. GenBank JF760180.1).

4.5 Proof-of-concept using gel electrophoresis

Gel electrophoresis was used to demonstrate the basic operation of our assay, as shown in Figure 4.2. The target sequence is synthetic DNA corresponding to HIV sequence. The target alone ran at a relatively low position in the agarose gel (arrow), while the CLAMP structures ran slightly higher. Upon mixing target and CLAMP structures together, we observed a smear corresponding to a wide distribution of product aggregate sizes. However, in the presence of an unrelated target, there was no interaction between the CLAMPS and the target. Thus, our hybridization-driven aggregation response was specific to the intended target sequence.

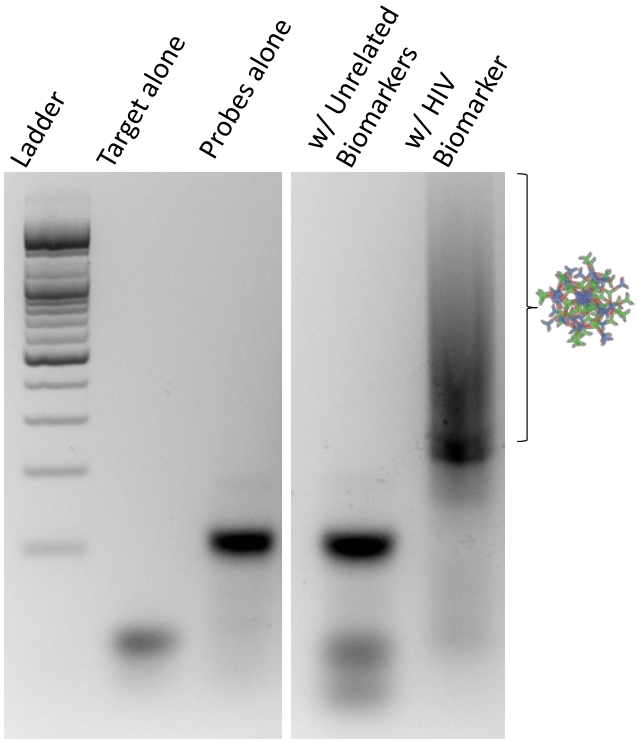


Figure 4.2: Gel electrophoresis showing proof of principle for our aggregation-based assay. Lane 1: 100 base-pair double-stranded DNA ladder. Lane 2: Target sequence (synthetic single-stranded DNA 50-mer with conserved sequence from HIV). Lane 3: Mixture of two different branched DNA probes. Lane 4: Mixture of branched DNA probes with an unrelated DNA target (conserved sequence from Influenza A).

4.5 Study of assay robustness

The above results were obtained by carrying out the aggregation assay under well-controlled laboratory conditions. These conditions are summarized in Table 4.3. Briefly, a sample containing the target sequence was added to a solution

containing our CLAMP structures and mixed gently by pipetting. The mixture was incubated at room temperature for one hour without further treatment or handling. The room temperature was controlled at 20 °C. The solutions were buffered against acid/alkaline conditions and maintained at pH 7.4. The solutions were prepared using ultrapure autoclaved water. After the one hour incubation, samples were immediately characterized using gel electrophoresis. In summary, these typical laboratory conditions were not necessarily representative of harsh uncontrolled conditions that may be experienced at the point of care in a resource-limited setting. Thus, we carried out additional sets of experiments to verify the robustness of our process in the context of various disturbances to our established experimental procedure as well as the presence of various contaminating interferences within the test samples.

Ideal (non-POC) conditions for hybridization based aggregation process	
Reaction conditions	Simple mixing, no shaking
Temperature	20 °C
Hybridization duration	1 hr
Water quality	Ultrapure autoclaved water
Acidity/alkalinity	Buffered at pH 7.4

Table 4.3: Summary of conditions used for the "best case" aggregation experiment, prior to testing with more challenging sample conditions as discussed below.

We sought to confirm the performance of our system across a range of non-ideal procedural conditions. First, we varied the operating temperature for the hybridization step, as shown in Figure 4.3. We observed that aggregation resulted in a consistent response even when the hybridization step was carried out above the theoretical melting temperature of the probe (approximately 55 °C). We speculate that this consistent behavior was due to enhanced binding offered by the polyvalent probes, which may have resulted in increased local concentrations of reactants and thus raising the effective melting temperature.

Secondly, we varied the duration of the hybridization steps. We observed the aggregation occurred for all the time spans tested, surprisingly even within five minutes. This extremely rapid hybridization could be partially explained by the high concentration of reactants in the sample (1 μM for each species of CLAMP structure), and lower concentrations were expected to result in slower kinetics with a yield of aggregate product that was highly dependent on the duration of hybridization. We also tested a range of annealing programs but did not observe any difference in the aggregation behavior for these samples.

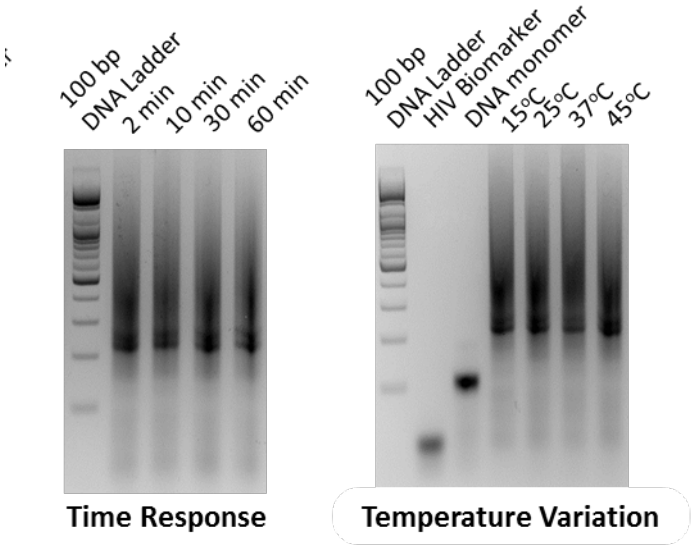


Figure 4.3: Gel electrophoresis to show effect of varying duration and temperature for the aggregation process. Results indicate aggregation occurs under both conditions.

To explore the possibility of using impure water samples as diluent for our assay, we obtained water from a variety of unconventional sources (including unpurified tap water, river water, and rain water) and carried out our assay within these samples. For all water conditions tested, we did not observe any change in the assay performance, though the presence of particulates in the water slightly affected the resulting smear in the gel electrophoresis image. We also adjusted the pH in our samples over a very broad range, from pH 3 to pH 11, and observed aggregation in all samples except for the pH 3 sample, in which the DNA appeared to be degraded (the DNA was likely depurinated under this extreme condition, some references give pH 4 as the threshold below which DNA is typically degraded).

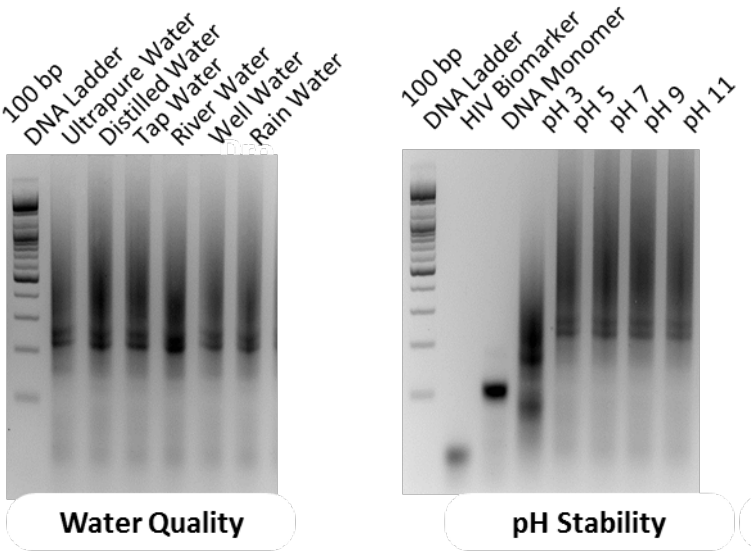


Figure 4.4: Gel electrophoresis to demonstrate the effect of varying water quality and pH on the sample results. Despite the impure water quality and varying pH, the aggregation process remained unaffected. For the pH of 3, DNA samples were degraded, so this condition is unsuitable for our assay.

In addition to testing procedural disturbances, we also considered the potential for sample contamination. This was important because the sample preparation process might be less clean at the point of care, and thus POC samples may tend to contain more contaminants than samples prepared in a conventional laboratory setting. We tested for this possibility by preparing samples spiked with various common contaminants, as shown in Figures 4.5 and 4.6. In particular, we spiked samples with various concentrations of red blood cells,

human serum, sodium chloride, and Hank's buffered saline solution. Again we found that the aggregation process was extremely reliable, such that none of the contaminants interfered with our assay.

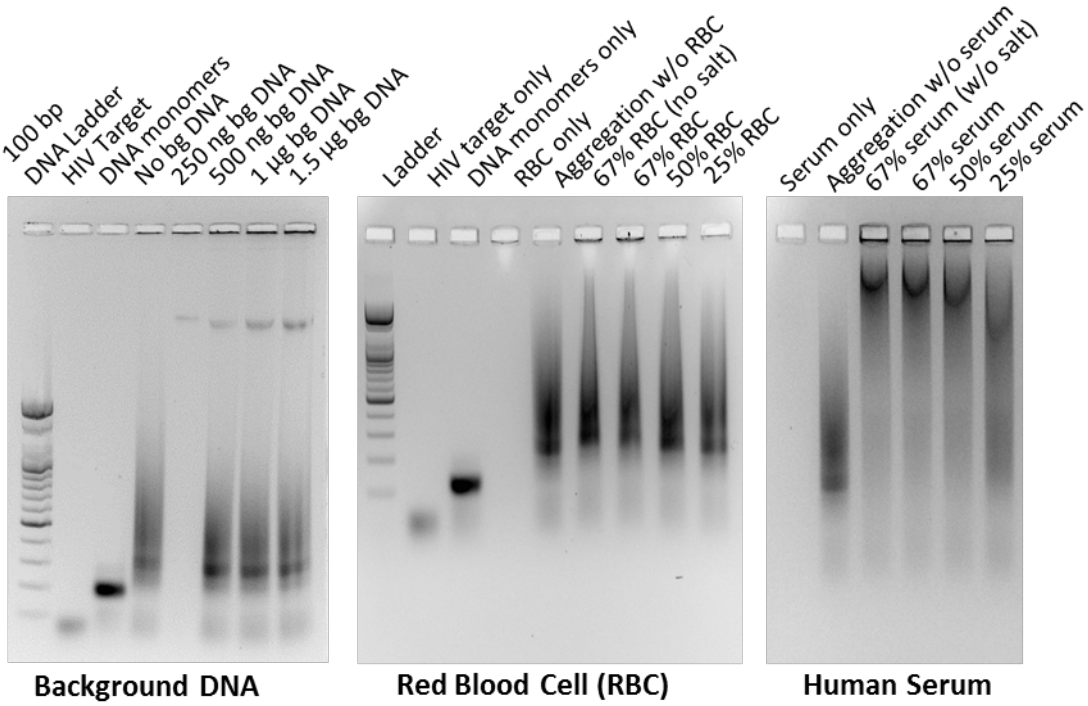


Figure 4.5: Gel electrophoresis demonstrating aggregation process in the presence of sample contamination. Background DNA does not interfere with the aggregation process. Red blood cells result in some disruption of the electrophoresis running pattern, possibly due to aggregation of blood cells in the well, but otherwise the aggregation process is unaffected. Human serum seems to result in some increased smearing of gel electrophoresis bands, but aggregation product is still clearly visible.

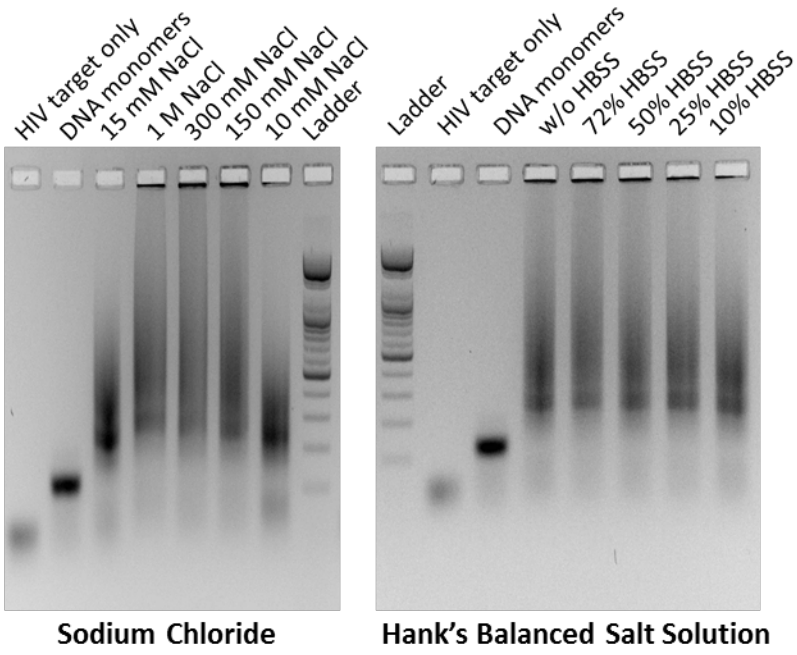


Figure 4.6: Gel electrophoresis showing aggregation process under different sodium chloride and Hank's Balanced Salt Solution (HBSS) concentrations. Limited aggregation was observed for 15 and 10 mM NaCl concentrations, since the sodium cation concentration was too low to facilitate hybridization.

Overall, through the aforementioned experiments, the aggregation process utilized in our assay was shown to be very robust over a fairly broad range of conditions. This unexpected result seems reasonable when we consider that hybridization itself is a very robust process, so our aggregation process based on hybridization was naturally robust as well.

4.7 Electrochemical detection

To achieve more sensitive detection with practical implications for POC testing, we also tested the detection of hybridization-driven aggregates using an electrochemical sensor.[20], [21] [20], [21] The sensor was capable of discriminating between aggregates and Y-shaped DNA based on dielectric (impedance) spectroscopy. This sensor has excellent advantages for POC detection, as shown in Table 4.4: it has a small compact size (important for portability), monolithic integration (no moving or fragile components), low power requirement (the CMOS-based platform is extremely efficient, potentially requiring only a small battery), low cost using established manufacturing processes, and consistent with existing CMOS standards enabling future integration with existing electronic devices.

Small size	Millimeter to centimeter scale, lightweight and portable
Stability	Monolithic architecture, robust with no fragile or moving components
Low power requirement	CMOS consumes power only to switch states
Cost efficient manufacturing	Highly scalable foundry-based production
Integration with components	Existing standards guarantees simple interfacing with additional CMOS features

Table 4.4: Brief list of advantages for using CMOS electrochemical sensor for point-of-care detection, relative to other candidate sensor technologies such as optical or mechanical sensors.

The device works similar to a traditional ion-sensitive field effect transistor (ISFET), in which current flows from source to drain and is modulated by a sensing gate. In a modification of the traditional ISFET design, Kan laboratory introduced an additional control gate, which allows the inputs to be weighted or thresholded. This control gate functions as a parallel input, with the inputs from the control gate and sensing gate both affecting the modulation of channel current. The ability to utilize weighted inputs allows the device to exhibit

neuron-like behavior, so this device is termed chemoreceptive neuron MOS, or CvMOS.

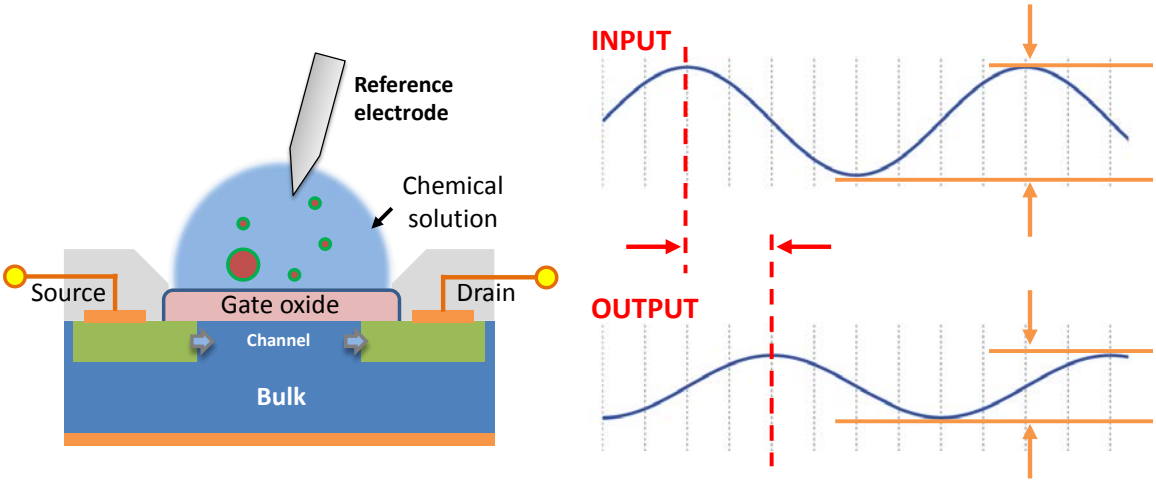


Figure 4.7: Basic principles of operation of ISFET and CvMOS in the mode of impedance (dielectric) spectroscopy.

This sensor was utilized for dielectric spectroscopy, which is a mode of measurement for characterizing the behavior of a medium in response to an alternating current. A probe was brought into contact with the sample and an AC field was applied. The input signal and output signal are measured and the relative changes to magnitude and phase give a unique signature that is related to the impedance characteristics of the sample. Using this approach,

hybridization-driven aggregates show a unique response compared to YDNA alone.

We tested the CMOS electrochemical sensor in impedance spectroscopy mode with a set of samples with a range of target concentrations, and we sought to determine an estimate of the sensitivity limit for our system. Using this electrochemical detection approach, we determined the limit of detection for hybridization-driven aggregation was between 100 femtomolar and 50 picomolar.

4.8 Gold nanoparticles for enhanced sensitivity

Encouraged by our preliminary tests of limit of detection, we sought to boost the sensitivity even further. In order to further exaggerate the difference between samples containing DNA aggregates (test positive results), and those containing no aggregates (test negative results), we determined it would be helpful to add gold nanoparticles as an additional component of the assay. Gold nanoparticles are a popular choice for working with DNA, and we had prior experience with DNA-capped gold nanoparticles in our group. Utilizing thiol-modified oligonucleotides, we prepared gold nanoparticles coated with capture probes that could recognize specific regions within the intended target sequences.

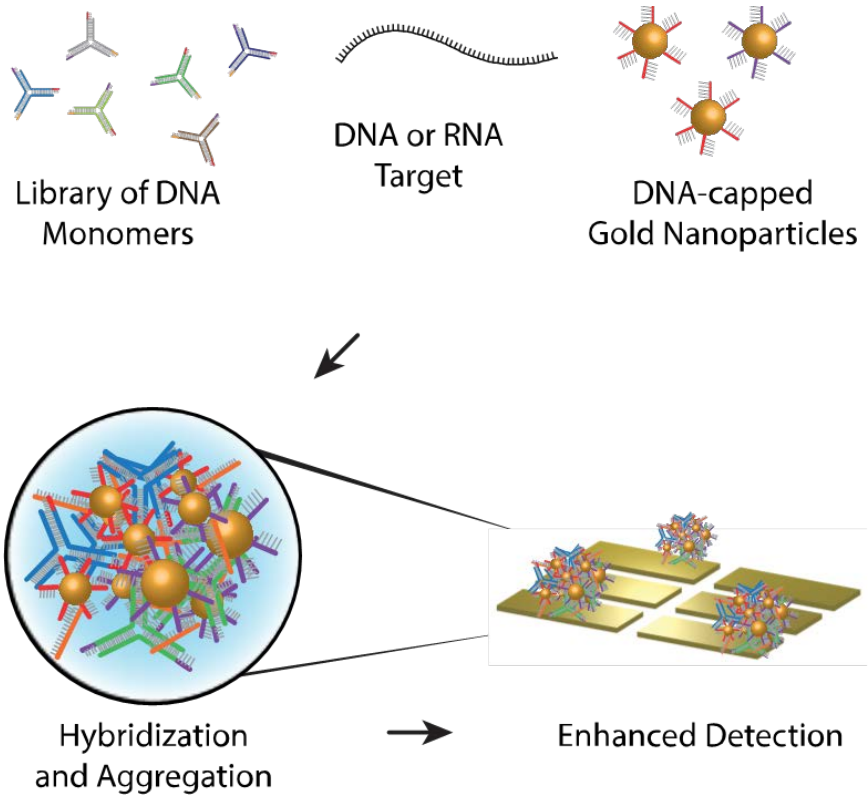


Figure 4.8: Scheme illustrating the use of gold nanoparticles with branched polyvalent probes for enzyme-free nucleic acid detection. The addition of gold nanoparticles to our assay was expected to result in enhanced sensitivity and reduced limit of detection.

For our first trial, we used bright field microscopy for testing our aggregation with DNA-capped gold nanoparticles. We observed obvious aggregation within the samples containing gold nanoparticles with DNA probes and target sequences. We further prepared a "scaled-up" version of this reaction and we

could clearly see the aggregates precipitate out of the solution over a duration of a few hours. We could also observe aggregation over shorter time scales using dynamic light scattering (DLS). These preliminary results demonstrated that the addition to gold nanoparticles did not interfere with our aggregation process and could potentially enable new modes for detection.

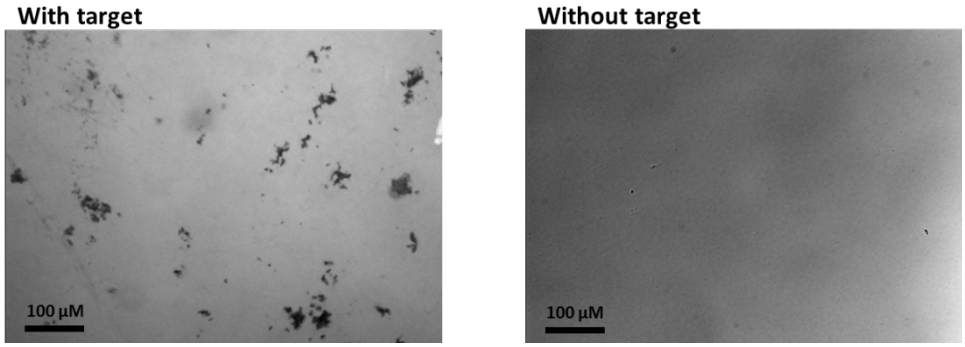


Figure 4.9: Optical microscopy of gold nanoparticle aggregates. Target-driven hybridization results in obvious aggregation of gold nanoparticles into dark clusters.

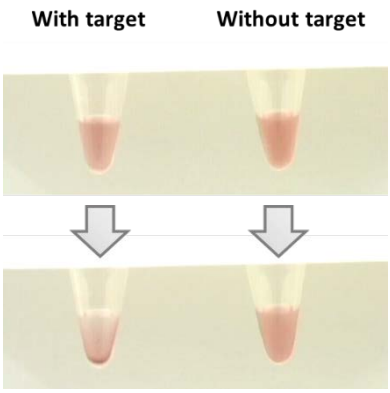


Figure 4.10: Naked eye detection of gold nanoparticle aggregates. Aggregation results in precipitation, which removes color from the solution.

Encouraged by these results, we further prepared samples for testing with the electrochemical sensor. We prepared a range of target concentrations, and carried out impedance spectroscopy measurements as previously described above. We found that the gold nanoparticles did in fact improve the sensitivity, allowing for highly sensitive results even at low target concentrations. We observed a response down to 100 femtomolar, which is almost hundred-fold improvement over our previous limit of detection, and superior to many established diagnostic methods. For example, the popular method enzyme-linked immunosorbent assay (ELISA) has a limit of detection of approximately 10 picomolar; furthermore, ELISA uses enzymes whereas our assay is enzyme-free and hence much more suitable for POC detection.

4.9 Conclusion and outlook

In conclusion, we demonstrated a streamlined aggregation process that is suitable for minimally complex detection. Importantly, this process is entirely enzyme-free, avoiding the limitations associated with using enzymes at the point-of-care. We have demonstrated this process is highly specific and robust against both procedural disturbances and sample contamination, which is of critical importance for establishing feasibility to use our assay at the point-of-care. We further demonstrated the detection of our aggregates using a POC-friendly electrochemical sensor. We used this system in a preliminary feasibility study to uniquely identify a target DNA sequence from HIV down to 50 picomolar concentration. By implementing gold nanoparticles into our assay, we further increased our sensitivity and reduced our detection limit down to 100 femtomolar concentration.

4.9 References

[1] M. Urdea, L. A. Penny, S. S. Olmsted, M. Y. Giovanni, P. Kaspar, A. Shepherd, P. Wilson, C. A. Dahl, S. Buchsbaum, G. Moeller, and D. C. Hay Burgess, "Requirements for high impact diagnostics in the developing world," Nov. 2006.

[2] S. Banoo, D. Bell, P. Bossuyt, A. Herring, D. Mabey, F. Poole, P. G. Smith, N. Sriram, C. Wongsrichanalai, R. Linke, R. O'Brien, M. Perkins, J.

Cunningham, P. Matsoso, C. M. Nathanson, P. Olliaro, R. W. Peeling, and A. Ramsay, "Evaluation of diagnostic tests for infectious diseases: general principles.," *Nat Rev Microbiol*, vol. 4, no. 12 Suppl, pp. S20–32, Dec. 2006.

[3] G. A. Posthuma-Trumpie, J. Korf, and A. van Amerongen, "Lateral flow (immuno)assay: its strengths, weaknesses, opportunities and threats. A literature survey.," *Analytical and Bioanalytical Chemistry*, vol. 393, no. 2, pp. 569–82, Jan. 2009.

[4] B. Ngom, Y. Guo, X. Wang, and D. Bi, "Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review.," *Analytical and Bioanalytical Chemistry*, vol. 397, no. 3, pp. 1113–35, Jun. 2010.

[5] R. M. Dirks and N. A. Pierce, "Triggered amplification by hybridization chain reaction.," *Proc Natl Acad Sci U S A*, vol. 101, no. 43, pp. 15275–8, Oct. 2004.

[6] D. Y. Zhang, A. J. Turberfield, B. Yurke, and E. Winfree, "Engineering entropy-driven reactions and networks catalyzed by DNA.," *Science*, vol. 318, no. 5853, pp. 1121–5, Nov. 2007.

[7] D. Y. Zhang and E. Winfree, "Control of DNA strand displacement kinetics using toehold exchange.," *J Am Chem Soc*, vol. 131, no. 47, pp. 17303–14, Dec. 2009.

[8] P. B. Allen, S. A. Arshad, B. Li, X. Chen, and A. D. Ellington, "DNA circuits as amplifiers for the detection of nucleic acids on a paperfluidic platform.," *Lab Chip*, vol. 12, no. 16, pp. 2951–8, Aug. 2012.

[9] X. Chen, N. Briggs, J. R. McLain, and A. D. Ellington, "Stacking nonenzymatic circuits for high signal gain," *Proceedings of the National Academy of Sciences*, Mar. 2013.

[10] C. A. Mirkin, R. L. Letsinger, R. C. Mucic, and J. J. Storhoff, "A DNA-based method for rationally assembling nanoparticles into macroscopic materials.," *Nature*, vol. 382, no. 6592, pp. 607–9, Aug. 1996.

[11] P. H. Rogers, E. Michel, C. A. Bauer, S. Vanderet, D. Hansen, B. K. Roberts, A. Calvez, J. B. Crews, K. O. Lau, A. Wood, D. J. Pine, and P. V. Schwartz, "Selective, Controllable, and Reversible Aggregation of Polystyrene Latex

Microspheres via DNA Hybridization," *Langmuir*, vol. 21, no. 12, pp. 5562–5569, Jun. 2005.

[12] Y. Wang, Y. Wang, D. R. Breed, V. N. Manoharan, L. Feng, A. D. Hollingsworth, M. Weck, and D. J. Pine, "Colloids with valence and specific directional bonding.," *Nature*, vol. 491, no. 7422, pp. 51–5, Nov. 2012.

[13] S. J. Tan, M. J. Campolongo, D. Luo, and W. Cheng, "Building plasmonic nanostructures with DNA.," *Nature Nanotechnology*, vol. 6, no. 5, pp. 268–76, May 2011.

[14] C. Woodbury, *Introduction to Macromolecular Binding Equilibria*. Boca Raton, FL: CRC Press, 2008.

[15] J. SantaLucia and D. Hicks, "The thermodynamics of DNA structural motifs.," *Annu Rev Biophys Biomol Struct*, vol. 33, pp. 415–40, Jan. 2004.

[16] J. B. Lee, Y. H. Roh, S. H. Um, H. Funabashi, W. Cheng, J. J. Cha, P. Kiatwuthinon, D. A. Muller, and D. Luo, "Multifunctional nanoarchitectures from DNA-based ABC monomers.," *Nature Nanotechnology*, vol. 4, no. 7, pp. 430–6, Jul. 2009.

[17] G. J. Tsongalis, "Branched DNA technology in molecular diagnostics.," *American Journal of Clinical Pathology*, vol. 126, no. 3, pp. 448–53, Sep. 2006.

[18] A.-C. Lee, Z. Dai, B. Chen, H. Wu, J. Wang, A. Zhang, L. Zhang, T.-M. Lim, and Y. Lin, "Electrochemical branched-DNA assay for polymerase chain reaction-free detection and quantification of oncogenes in messenger RNA.," *Analytical Chemistry*, vol. 80, no. 24, pp. 9402–10, Dec. 2008.

[19] A. Rambaut, D. L. Robertson, O. G. Pybus, M. Peeters, and E. C. Holmes, "Human immunodeficiency virus. Phylogeny and the origin of HIV-1.," *Nature*, vol. 410, no. 6832, pp. 1047–8, Apr. 2001.

[20] B. C. Jacquot, N. Muñoz, D. W. Branch, and E. C. Kan, "Non-Faradaic electrochemical detection of protein interactions by integrated neuromorphic CMOS sensors.," *Biosens Bioelectron*, vol. 23, no. 10, pp. 1503–11, May 2008.

[21] N. Y. Shen, Z. Liu, C. Lee, B. A. Minch, and E. C. Kan, "Charge-based chemical sensors: a neuromorphic approach with chemoreceptive neuron MOS (C/sub V/MOS) transistors," *IEEE Transactions on Electron Devices*, vol. 50, no. 10, pp. 2171–2178, Oct. 2003.

CHAPTER 5

CONCLUSION AND OUTLOOK

One of the central goals of nanotechnology is the precise control and manipulation of matter on the nanometer scale. As the state of the art for design and synthesis of DNA nanostructures continues to advance, this goal is becoming increasingly easy to realize through DNA nanotechnology. For example, the use of DNA origami and molecular canvas approaches, as demonstrated in recent publications, have enabled unprecedented feats of nanometer-scale engineering. However, the practical significance of DNA nanostructures has been slow to materialize, and a real-world demonstration of DNA nanostructures as utilized for practical applications would be extremely valuable for motivating further advancement in this field.

One of the most direct and important applications for DNA nanostructures is in the area of molecular diagnostics. Nucleic acids obviously play a critical role in biological processes, but historically and traditionally have not played a leading role in the diagnostic clinical laboratory. Instead, cell culture and immunological-based tests have dominated due to their earlier development and precedence. For example, the first cell culture experiments were carried out in the 1800s, and cell

culture became a common laboratory technique in the mid-1900s,[1]. Practical diagnostic methods utilizing nucleic acids were not fully developed and adopted until recent decades (PCR was not developed until 1983)[2]. As the utility of nucleic acids as biomarkers is increasingly recognized, and as molecular biology and sequencing technologies continue to advance, molecular diagnostics are steadily growing in importance for both research and clinical applications. Molecular diagnostic techniques, including detection, expression profiling, and sequencing, are revolutionizing the standard of care for clinical prognostics and diagnostics.

Virtually all techniques in the area of molecular diagnostics techniques must make use of DNA as primers and/or probes. Even simple variations on the standard DNA primers and probes has led to a vast assortment of technologies, including molecular beacons, scorpion probes, bDNA assays, etc. Thus, the full range of possibilities offered by DNA nanotechnology offers incredible promise for the development of new and disruptive diagnostic technologies. The unique advantages that arise from DNA nanostructures, such as multifunctionality and controllability, could potentially have great benefits for molecular diagnostic techniques. However, the potential of branched DNA nanostructures has not been fully explored and utilized in these applications. This is a great opportunity for fundamental and applied research on DNA nanotechnology to integrate

DNA nanostructures into molecular diagnostic applications, before current practices become widely-adopted and standardized.

Chapter 2 described an approach to overcome current limitations or concerns about the stability of DNA nanostructures. Simple photo-cross-linking treatment using psoralen, an inexpensive and naturally-occurring chemical, can generate DNA nanostructures with thermostable property. The enhanced stability of these structures was confirmed under various denaturing conditions, and it was shown that this method was general and could be applied to any branched DNA nanostructure design. The thermostable structures were then utilized in PCR (as "modular primers") and the resulting PCR products showed that these "modular primers" resulted in various PCR products that were impossible to achieve in conventional PCR with linear primers. For example, the use of fluorophore-labeled modular primers could allow the "DNA nanobarcodes" concept to work with enhanced sensitivity (theoretically improving the sensitivity limit to that of PCR), using multiplexed PCR to carry out simultaneous amplification and multiplexed labeling of products. Furthermore, modular primers containing multiple linear primers on a single branched structure enabled the production of multi-armed branched PCR products which could be used for the delivery of controlled ratio of genes. In addition, a DNA hydrogel produced through PCR of multiple branched primers was demonstrated, with applications for cell free

protein expression. In summary, a varied assortment of novel PCR products were produced from the same straightforward approach, demonstrating the power and versatility of applying DNA nanostructures to an established molecular biology technique. The focus of this work was on PCR, but the enhanced stability of thermostable DNA nanostructures could be beneficial for many other important applications that involve potentially denaturing conditions.

Chapter 4 described empirical studies of a general system that is fundamental for DNA nanostructures: hybridization-driven aggregation. Whereas many assays utilize immunologically-based agglutination, nucleic acid-based agglutination is rarely considered as a feasible approach for detection. Our approach utilized branched DNA nanostructures with multiple capture probes on each single structure. As a result, each probe could bind to multiple targets and each target could bind to multiple probes. Our experiments indicated that individual DNA hybridization events could result in aggregation and lead to very high local concentration of DNA. This response was shown to be specific, occurring only in the presence of a specific target DNA sequence in conjunction with probes that were designed to be complementary. We further demonstrated that this response was robust and could be carried out under a broad range of reaction conditions, both by varying procedural parameters for the aggregation process and by

testing contaminated samples spiked with common interferents. We utilized an electrochemical sensor for the detection of the resulting aggregates, demonstrating a potentially viable strategy for point of care detection in resource limited settings. To demonstrate the feasibility of POC diagnostics using this platform, we utilized an electrochemical sensor for measurement of the resulting hybridization-driven aggregates, achieving a detection limit down to 50 picomolar. This entire system is compatible with POC diagnostics and could be extremely valuable for resource limiting settings such as the developing world. In addition to detection, this work was useful as a fundamental study of DNA-based self-assembly and hybridization-driven polymerization.

The works described in this dissertation sought to demonstrate the feasibility and advantages of DNA nanostructures in molecular diagnostics, with the aim to strengthen claims of practical significance for DNA nanotechnology. Future work in this area could aim to achieve real-world implementation and focus on utilizing the platform technologies describe here in conjunction with more realistic sample matrices and environmental conditions. With further development, the incorporation of DNA nanostructures into existing and novel strategies for molecular diagnostics will ultimately lead to advancements in diagnostics and improved clinical outcomes.

References

[1] "Molecular Biology of the Cell." Garland Science, 2002.

[2] J. M. S. Bartlett and D. Stirling, "A short history of the polymerase chain reaction.," *Methods Mol Biol*, vol. 226, pp. 3–6, Jan. 2003.